Vertical microbial transmission in Caribbean bacteriosponges

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Erklärung

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Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe.

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Ich erkläre weiterhin, dass ich früher weder akademische Grade erworben habe, noch versucht habe zu erwerben.

Würzburg, April 2007

(Susanne Schmitt)

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JOHANNA UND MORITZ RABENSTEIN

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UND FÜR MEINE MUTTER

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Summary

Bacteriosponges contain large amounts of morphologically and phylogenetically diverse microorganisms in their mesohyl. The association is permanent, stable and highly specific, however, little is known about the establishment and maintenance of this association. The first aim of this Ph.D. thesis was to examine cospeciation between eight *Aplysina* species from the Mediterranean and Caribbean and their cyanobacterial associates. Host phylogeny was constructed with 18S rDNA and ITS-2 sequences using an alignment based on the secondary structure of the molecular markers and five different algorithms each. The genus *Aplysina* appeared as monophyletic. *Aplysina* sponges could be distinguished into a Caribbean and a Mediterranean cluster and a possible Tethyan origin is suggested. Comparison of the host phylogeny to the 16S rDNA phylogeny of the cyanobacterial strains revealed the lack of a congruent pattern. Therefore it is proposed that *Aplysina* sponges have not cospeciated with their cyanobacterial phylotypes and probably also not with other sponge specific microbes.

The second aim of this Ph.D. thesis was to examine vertical transmission of microorganisms through reproductive stages of sponges. A general transmission electron microscopy (TEM) suvey revealed a clear correlation in that bacteriosponges always contained many microorganisms in their reproductive stages whereas non-bacteriosponges were always devoid of microbes in their reproductive stages. The transmission of the microbial community via sponge reproductive stages is concluded.

Based on the previous results *Ircinia felix* was chosen for a detailed documentation of vertical transmission. *I. felix* larvae contained large amounts of microorganisms extracellularly in the central region whereas the outer region was almost free of microbes as shown by TEM. In *I. felix* juveniles microorganisms were located between densely packed sponge cells. The microbial profiles of *I. felix* adult, larvae, and juveniles were compared using denaturing gradient gel electrophoresis (DGGE). Similar microbial community patterns were found in adult and the respective larvae indicating that a large subset of the adult microbial community was vertically transmitted. In contrast, microbial communities of larvae pools released by different adult individuals seemed to be more variable. Juvenile banding patterns were a mixture of sponge specific and seawater microbes due to DNA extraction artefacts but

demonstrated that at least half of the adult microbial community is present in the next generation.

Finally, a comprehensive phylogenetic analysis was conducted by sequencing excised DGGE bands from adult and offspring of the bacteriosponges *Agelas wiedenmayeri*, *I. felix*, and *Smenospongia aurea* and by taking additional 16S rDNA sequences of *Ectyoplasia ferox* and *Xestospongia muta* (unpublished data of the laboratory). The identification of 24 vertical transmission clusters in at least 8 eubacterial phyla demonstrates that a complex and uniform microbial community is transferred via sponge reproductive stages. Vertical transmission is specific in that the microorganisms of bacteriosponges, but not those from seawater, are passed on, but unselective in that there appears to be no differentiation between individual sponge-specific lineages. In conclusion, vertical transmission points to a mutualistic and long-term association of bacteriosponges and complex microbial consortia.

Zusammenfassung

Bakterienhaltige Schwämme sind durch große Mengen an morphologisch und phylogenetisch unterschiedlichen Mikroorganismen im Mesohyl gekennzeichnet. Diese mikrobiellen Konsortien sind permanent, stabil und hoch-spezifisch mit den Wirts-Schwämmen assoziiert. Über die Entstehung und die Aufrechterhaltung dieser Assoziation ist jedoch wenig bekannt. Es war das erste Ziel dieser Doktorarbeit, Co-Speziation zwischen mediterranen und karibischen Schwämmen der Gattung *Aplysina* und assoziierten Cyanobakterien zu untersuchen. Die Wirtsphylogenie wurde sowohl mit 18S rDNA als auch mit ITS-2 Sequenzen erstellt. Das Alignment basierte auf der Sekundärstruktur des jeweiligen molekularen Markers und jeder phylogenetische Stammbaum wurde mit 5 verschiedenen Algorithmen berechnet. Die Gattung *Aplysina* erschien monophyletisch. Die verschiedenen Arten konnten einer Karibik- und einer Mittelmeer-Gruppe zugeordnet werden und der Ursprung der Gattung *Aplysina* im Urmeer Tethys erscheint möglich. Der Vergleich von Wirts- und Cyanobakterien-Phylogenie, welche auf 16S rDNA Sequenzen beruht, zeigte, dass die Topologie der Stammbäume sich nicht spiegelbildlich gegenübersteht. Es wird daher angenommen, dass keine Co-Speziation zwischen *Aplysina* Schwämmen und Cyanobakterien und wahrscheinlich auch nicht mit anderen Schwamm-spezifischen Mikroorganismen vorliegt.

Das zweite Ziel dieser Doktorarbeit war, die vertikale Weitergabe von Mikroorganismen über Reproduktionsstadien in Schwämmen zu untersuchen. Eine umfangreiche elektronenmikroskopische Studie zeigte eine klare Korrelation, da bakterienhaltige Schwämme immer auch unterschiedliche mikrobielle Morphotypen in den Reproduktionsstadien aufwiesen, wohingegen in den Reproduktionsstadien bakterienarmer Schwämme keine Mikroorganismen gefunden wurden. Aus diesen Ergebnissen wird die Weitergabe des mikrobiellen Konsortiums über Reproduktionsstadien bakterienhaltiger Schwämme geschlossen.

Basierend auf den vorherigen Ergebnissen wurde *Ircinia felix* für eine detaillierte Dokumentation der vertikalen Weitergabe von Mikroorganismen ausgewählt. Elektronenmikroskopische Aufnahmen zeigten, dass die Larven von *I. felix* im zentralen

Bereich große Mengen an extrazellulären Mikroorganismen enthielten während der äußere Bereich nahezu frei von Mikroorganismen war. In *I. felix* Juvenilschwämmen waren die Mikroorganismen zwischen eng gepackten Schwammzellen lokalisiert.

Die mikrobiellen Profile von *I. felix* Adult, Larven und Juvenilen wurden mittels Denaturierender-Gradienten-Gel-Elektrophorese (DGGE) verglichen. Ähnliche mikrobielle Diversitätsmuster waren im Adultschwamm und den respektiven Larven vorhanden. Dies deutet darauf hin, dass ein großer Anteil des adulten mikrobiellen Konsortiums vertikal weitergegeben wird. Im Gegensatz dazu schienen die mikrobiellen Konsortien von Larven, die von unterschiedlichen Adultindividuen stammten, insgesamt variabler zu sein. Die Bandenmuster der Juvenilschwämme waren eine Mischung aus Schwamm-spezifischen und Seewassermikroorganismen, was auf die Methodik der DNA-Extraktion zurückgeführt werden kann. Allerdings kann gesagt werden, dass mindestens die Hälfte des adulten mikrobiellen Konsortiums in der nächsten Generation vorhanden war.

Schließlich wurde eine umfangreiche phylogenetische Analyse mit Sequenzen aus Adultschwämmen und Larven durchgeführt. Die Sequenzen wurden durch Sequenzierung von ausgeschnittenen DGGE-Banden der bakterienhaltigen Schwämme *Agelas wiedenmayeri*, *I. felix* und *Smenospongia aurea* gewonnen. Zusätzlich wurden bislang unveröffentlichte Sequenzen aus den Schwämmen *Ectyoplasia ferox* und *Xestospongia muta* verwendet, die im Labor erstellt worden waren. Die Identifizierung von 24 "vertical transmission clusters" in mindestens 8 verschiedenen, eubakteriellen Phyla zeigt, dass ein komplexes, aber einheitliches, mikrobielles Konsortium über die Reproduktionsstadien weitergegeben wird. Der Prozess der vertikalen Weitergabe ist spezifisch, da Mikroorganismen der bakterienhaltigen Schwämme, nicht aber Seewasser-Mikroorganismen weitergegeben werden. Zugleich scheint der Prozess der vertikalen Weitergabe nicht selektiv zu sein, da keine Unterscheidung zwischen einzelnen, Schwamm-spezifischen Mikroorganismen erfolgt. Insgesamt deutet vertikale Weitergabe auf eine mutualistische und seit langem bestehende Assoziation zwischen bakterienhaltigen Schwämmen und komplexen, mikrobiellen Konsortien hin.

Chapter One
General Introduction

I. Sponges (phylum Porifera)

Sponges, phylum Porifera, are an evolutionarily extremely successful group of animals. With 7,000 formally described and an estimated 15,000 extant species in three classes (Demospongiae, Calcarea, Hexactinellida) sponges are among the most diverse aquatic invertebrate phyla (Hooper and van Soest 2002). They are distributed worldwide in all aquatic habitats from marine shallow waters to the deep ocean, from warm tropical reefs to the cold polar seas and are present even in freshwater lakes and streams. Sponges developed a variety of shapes (ranging from thin encrusting, to vase-, fan-, ball-, or massive volcano-like forms), sizes (ranging from a few millimeters to >1m), and colors (due to the possession of pigments and/ or the presence of cyanobacteria ranging from inconspicuous black, brown, beige, or white to colourful yellow, orange, red, green or blue forms) (Fig. 1). Many sponges are morphologically plastic and the shape, size, and color may differ within one species depending on varying environmental factors such as light, current, and sedimentation, or on the sponge diet.

Palaeontological data revealed the very long geological history of the phylum Porifera. The fossil record starts with 580 million years (my) old skeleton spicules, soft body parts and embryos that were attributed to sponges and were found in deposits of the Early Vendian Doushantou phosphorites in China (Li et al. 1998). Gehling and Rigby (1996) affiliated the 565 my old organism *Palaeophragmodictya* of the Australian Ediacara-fauna with sponges. Sponge-related spicule remains were described from 555 my old *Cloudina*-reefs of southern Namibia (Reitner and Wörheide 2002) and from 543-549 my old Mongolian deposits (Brasier et al. 1997). These data demonstrate the existence of a widely distributed sponge fauna in Precambrian times, well before the sudden appearance of most extant animal phyla in the Cambrian (Cambrian explosion, ~545-535 my ago). The Precambrian existence is further supported by estimates based on molecular amino acid sequences of S-type lectins and of receptor tyrosine kinases that suggest an origin 800 and 650-665 my ago, respectively (Hirabayashi and Kasai 1993, Schäcke et al. 1994a). Chemofossils such as the sponge-

specific 24-isopropylcholestanes that were found in 1.8 billion years old stromatolites indicate an even much earlier origin (McCaffrey et al. 1994).

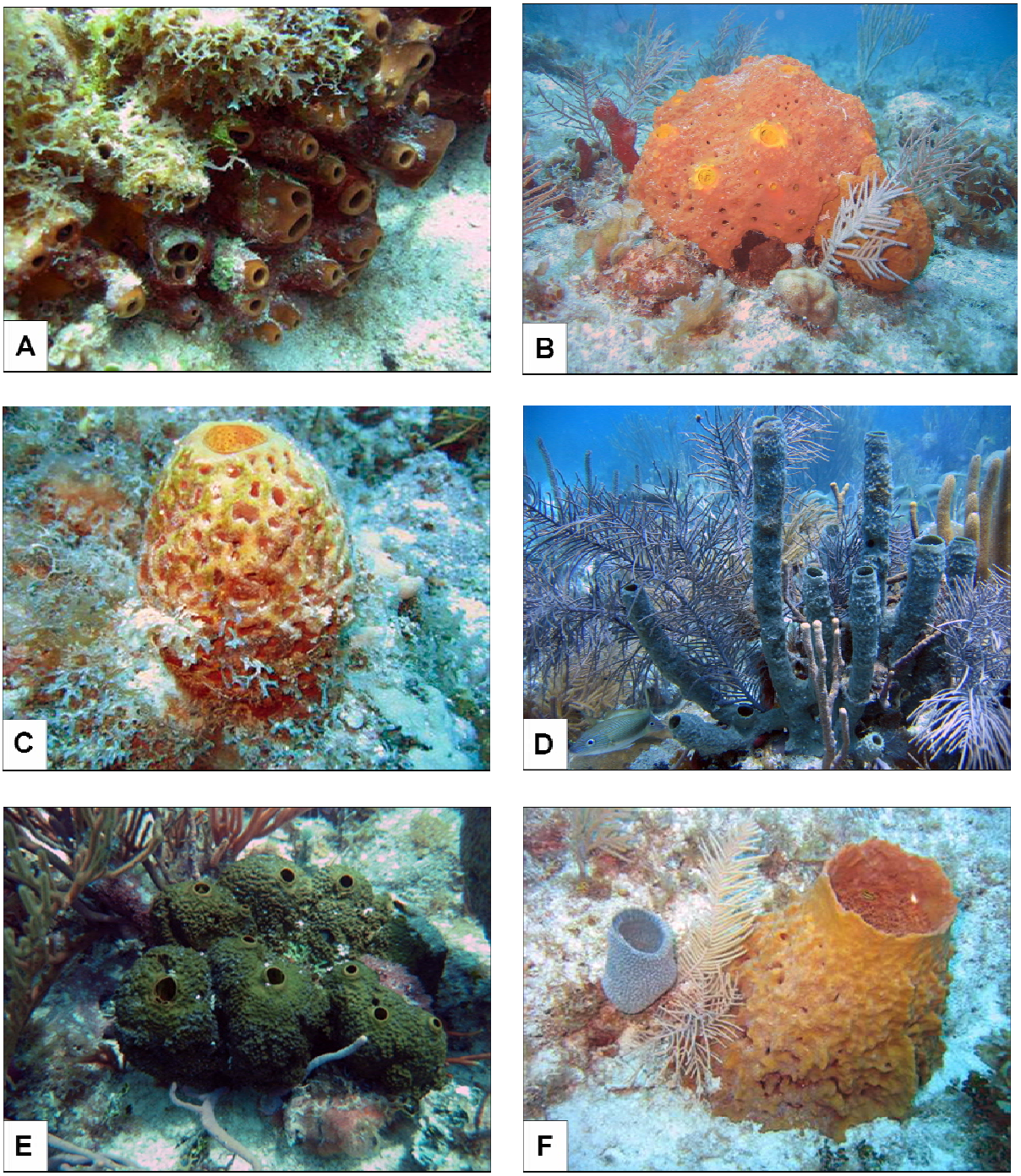

Fig. 1: A-F: Caribbean demosponges. (A): *Agelas wiedenmayeri*; (B): *Aiolochroia crassa*; (C): *Aplysina lacunosa*; (D): *Callyspongia vaginalis*; (E)*: Smenospongia aurea*; (F): *Niphates digitalis* (left), *Xestospongia muta* (right). Pictures by S. Schmitt.

From the Cambrian on, sponges were well established with all main clades present. After the late Devonian crisis, one of the biggest worldwide extinction events, the community of the long existing stem groups changed and the first modern groups appeared until all modern major taxa were present in the Permian. In the Mesozoic, sponges radiated enormously until they reached their greatest diversity in the Cretaceous. Also in the Mesozoic, probably in Early Jurassic, some marine taxa invaded freshwater environments as indicted by some well-preserved freshwater sponge remains similar to modern freshwater species (Volkmer-Ribeiro and Reitner 1991). Throughout most of the Palaeozoic and Mesozoic sponges were major reef builders, which formed for example the massive Devonian coral-stromatoporoid reefs or the large Upper Jurassic sponge-algal reefs of southern Germany (Stanley 1989). Today, hermatypic corals are the major contributors to reef formation but sponges remain ecologically very important reef-dwellers that sometimes even dominate the reef communities (Diaz and Rützler 2001).

Sponges possess the simplest body plan of all multicellular animals as they lack true organs or tissues and they are morphological conservative because they kept their fundamental body plan largely unchanged for million of years (Brusca and Brusca 1990, Hooper and van Soest 2002). The entire body structure is adapted to a filter-feeding life style and is characterized by the following features that are unique to sponges: First, choanocytes which are a special type of flagellated collar cells. Second, an aquiferous system that consists of inhalant and exhalant water canals as well as choanocyte chambers lined by choanocytes. The canal system is embedded in an extracellular matrix, termed the mesohyl and functions as a filter system. Unidirectional water current is generated by the coordinated beating of choanocyte flagella and pumped through the canals. The water current provides sponges with oxygen and nutrients, predominantly microorganisms, and it allows the expulsion of waste products. During evolution, the aquiferous system became more complex and consequently the filtration capacity continuously increased. Third, amoeboid-like sponge cells, termed archaeocytes that are highly mobile and move freely through the mesohyl. These cells are totipotent and can differentiate into other cell types with different functions such as fibre producing collencytes, spicule producing sclerocytes, and spongin producing spongocytes. Totipotency is the reason for the remarkable regenerative and reconstitutive ability of sponges. Fourth, the inorganic skeleton consisting of single calcite or silicate elements, termed spicules. Together with the organic spongin and collagen fibres, the spicules provide and keep the firm structure of the sponge body.

Despite the morphological simplicity, sponges possess a gene repertoire whose homologs in higher animals are involved in essential processes of multicellular development and morphogenesis. One class of such genes encodes cell signaling molecules. Cell signaling pathways are important in the regulation of development and in recognition and response to extracellular stimuli (Pires-daSilva and Sommer 2002). The receptor tyrosine kinase was detected in the sponge *Geodia cydonium* already more than ten years ago (Schäke et al. 1994b, Kruse et al. 1997). Recently, homologous core genes of six of the seven major animal signaling pathways were described from the sponge *Oscarella carmela* (Nichols et al. 2006), revealing an unexpected diversity of signaling genes in sponges. Another class of genes with homologs in sponges are the cell adhesion genes that are responsible for the diversification of the animal body plan by regulating the migration of cell population and the formation of body compartments through epithelia (Gumbiner 1996). Surprisingly, sponge homologs of the majority of animal cell-adhesion gene families were found, among them genes for cell-surface receptors, extracellular matrix proteins, and cytoskeletal-linker proteins (Nichols et al. 2006, Segawa et al. 2006). It is currently unknown why sponges have the genetic potential for a higher complexity but kept their simple morphology since the Precambrian. It was proposed that early animals evolved intricate developmental programs long before they evolved diverse developmental outcomes and that pre-existing gene families developed new interactions that conferred new functions to the ancestor of higher animals (Nichols et al. 2006).

Sponge systematics is based on a few reliable morphological characters. For example, cytological features such as the ultrastructure and distribution of choanocytes or the shape of choanocyte chambers are used particularly in higher taxonomic levels. Chemistry, size, geometry, ornamentation, and distribution of spicules are key characters for the identification and classification of sponges from class to species level. However, because of the paucity of morphological characters and the plasticity of sponges, their taxonomy is often problematic and is permanently under revision. It is therefore necessary to apply additional techniques such as the construction of phylogenetic trees with molecular sequences to elucidate relationships within the Porifera. A range of DNA markers (ribosomal, mitochondrial, protein coding gene sequences) with different resolution potential has been tested and many were found to be appropriate in phylogenetic studies (reviewed in Borchiellini et al. 2000, van Oppen et al. 2002). To date, most of these studies concentrated on deeper phylogenies such as relationships of poriferan classes or metazoan groups including sponges (e.g. Adams et al. 1999, Borchiellini et al. 2001, McInerney et al. 1999, Medina et al. 1998). In contrast, studies

on phylogeny, biogeography, or population structure of specific sponge groups are still scarce (e.g. Alvarez et al. 2000, Duran et al. 2004, Schmitt et al. 2005, Wörheide et al. 2002) although an increasing need of such studies exists.

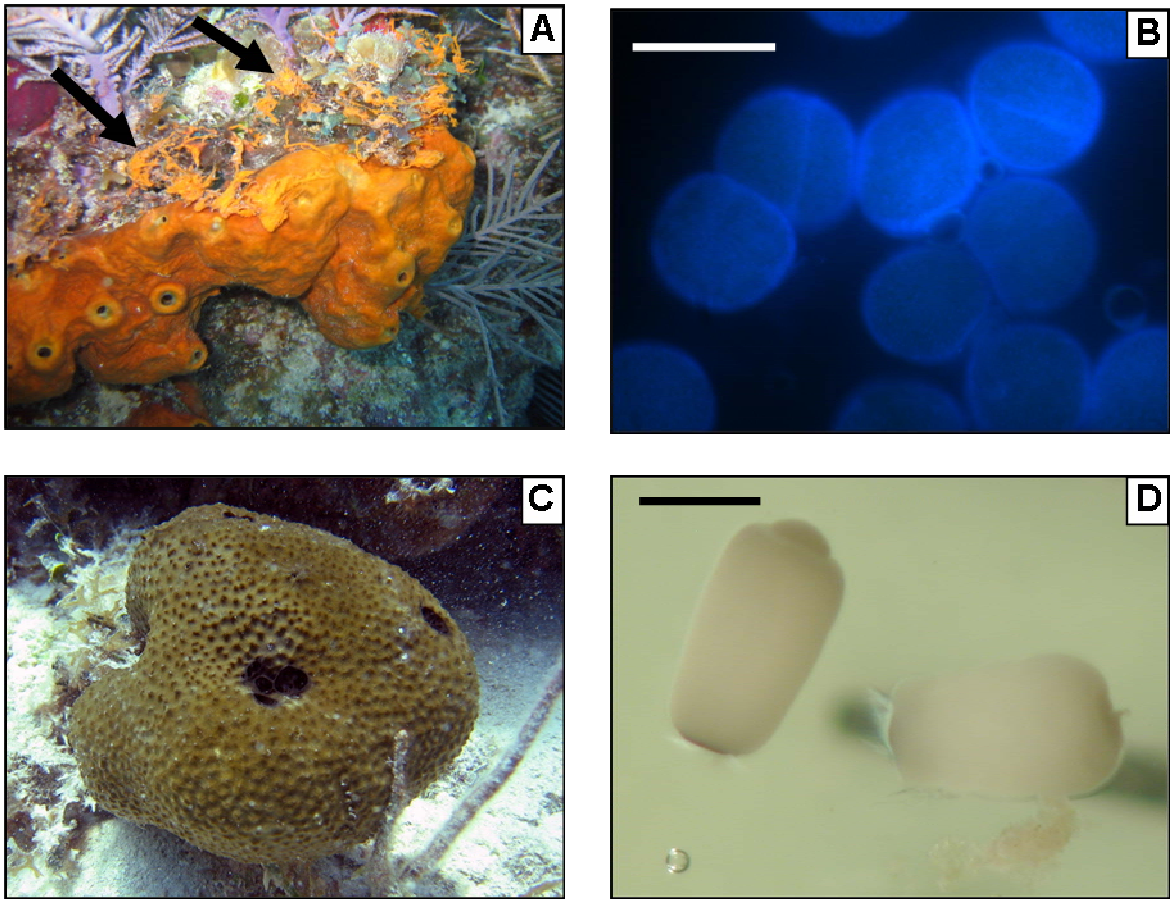

Fig. 2: A-B: The oviparous species *Ectyoplasia ferox* (A) and light microscopy of its oocytes (B). Arrows indicate the mucus sheath in which oocytes are embedded. C-D: The viviparous species *Ircinia felix* (C) and light microscopy of its larvae (D). Scale bar: 120µm (B), 250µm (D). Pictures by S. Schmitt (A, C), H. Angermeier (B), and N. Lindquist (D).

Reproduction within the phylum Porifera is a variable process that can differ considerably from species to species (Brusca and Brusca 1990). Because of the lack of true reproductive organs, sexual reproduction occurs in the mesohyl where gametes are produced by archaeocytes. Many species are hermaphroditic and oocytes and sperm are produced by the same specimen although often asynchronously. In contrast, gonochoric sponges have separate sexes and oocytes and sperm are produced by female and male specimens, respectively. The periods of gamete development also vary between different species and can last up to several months (Fromont and Bergquist 1994). Sponges with an oviparous mode of reproduction

release gametes into the water column whereby the oocytes are often embedded in a fine mucus-like sheath that adheres to the adult sponges and may increase the chance of fertilization (Fig. 2A, B). In contrast, viviparous sponges have an internal fertilization and brood embryos in the mesohyl, sometimes in special brooding chambers, until larvae are released into the seawater (Fig. 2C, D). The mobile larvae actively swim or creep for some hours to few days until they find a suitable substrate for settlement. During metamorphosis and the rearrangement of the body structure, the aquiferous system is established and the juvenile sponge switches over to the sessile filter-feeding life phase. In addition to sexual reproduction, many sponges also reproduce asexually by budding, fragmentation, or the production of over wintering gemmulae.

II. Sponge associated microbial consortia

When sponges first appeared 580 my ago, they were confronted with a microbial complexity that had already developed for over 3 billion years. Since then different kinds of interactions between sponges and microbes have evolved. Beside trophic and pathogenic (Olson et al. 2006, Webster et al. 2002) interactions also commensal or even symbiotic associations exist today. One example is the symbiosis between methane-oxidizing bacteria and the sponge *Cladorhiza methanophila* (Vacelet et al. 1995, 1996). This deep-sea carnivorous sponge is associated with two different types of bacteria of which at least one is methanotrophic. Although the sponge is still able of capturing small prey it seems to be nutritionally reliant on its methanotrophic symbionts. A second example is the association of filamentous cyanobacterial *Oscillatoria spongeliae* strains and four dictyoceratid sponge species (families Thorectidae and Dysideidae) (Berthold et al. 1982, Ridley et al. 2005a, Thacker and Starnes 2003). Although the bacteria are morphologically and phylogenetically similar each sponge species contains a distinct strain responsible for the different secondary metabolite composition in each species. An investigation on the total diversity in these four species revealed that other bacteria are only minor components and most of them are probably transient seawater bacteria (Ridley et al. 2005b).

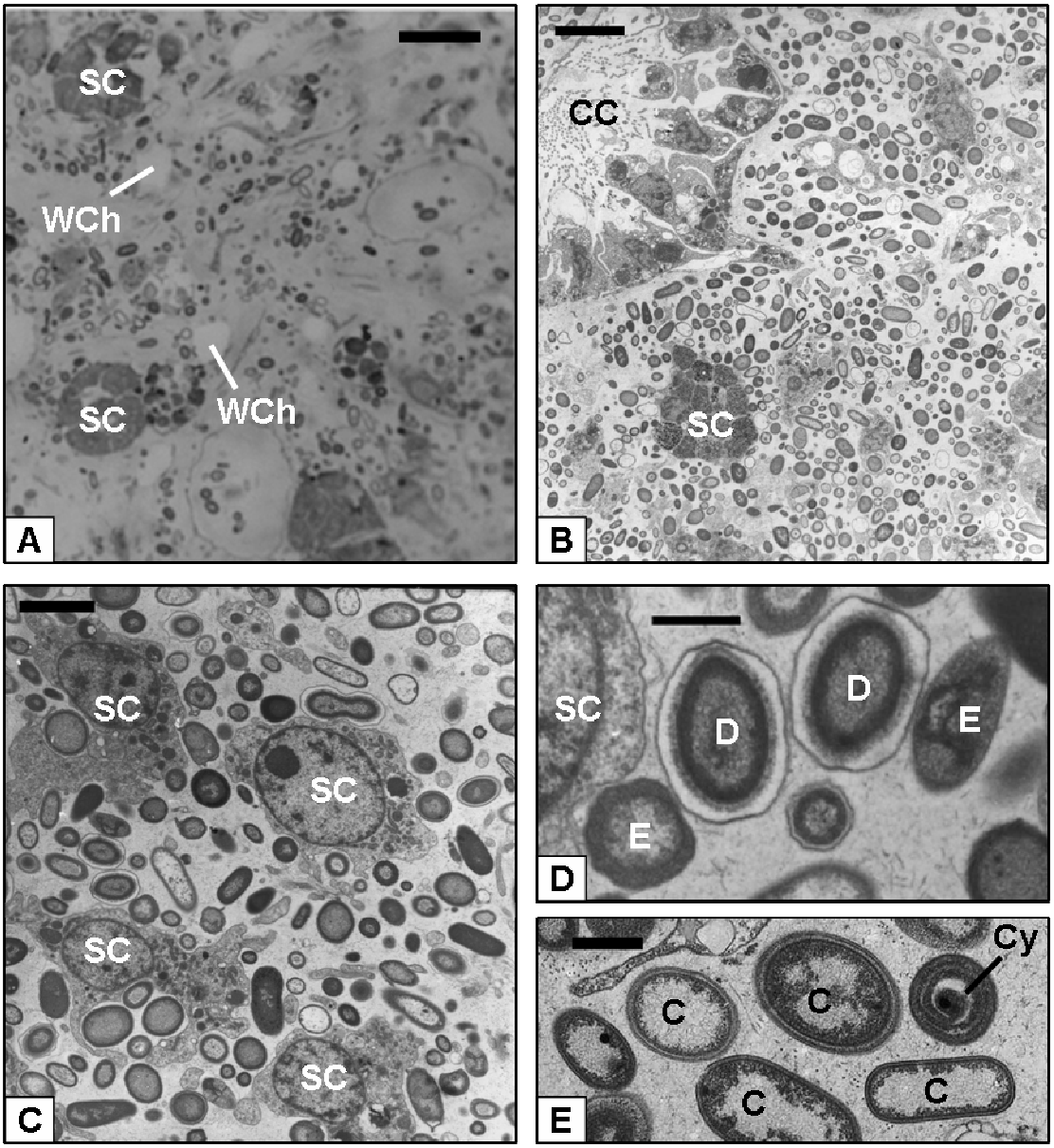

Fig. 3: Light (A) and electron (B-E) microscopy of the mesohyl of *Aplysina aerophoba*. A, B, and C provide an overview of microbial abundances and distribution (increasing magnification). D and E illustrate the previously described microbial morphotypes C, D and E (Friedrich et al. 1999). CC: Choanocyte chamber; SC: Sponge cell; WCh: Water channel. Scale bar: 10µm (A), 5µm (B), 1µm (C), 0.5µm (D, E). Pictures by S. Schmitt (A, E) and C. Gernert (B, C, D).

Another example is the permanent association of demosponges with microbial consortia (Hentschel et al. 2003, 2006, Lee et al. 2001) that is remarkable in several aspects. The microorganisms are present in high concentrations throughout the mesohyl as has been shown by electron microscopy (Friedrich et al. 1999, Wehrl 2006) (Fig. 3A-C). Often the microbes are equally scattered although clustered microbial accumulations can also be found. The extracellular microorganisms surround sponge cells and water canals but they seem not to be

taken up and digested by sponge cells. Quantitative studies revealed microbial concentrations of 10^8-10^{10} cells g^{-1} sponge weight (Friedrich et al. 2001, Webster and Hill 2001, U. Hentschel, pers. communication) and in some sponge species microorganisms amount up to 40% or even 60% of the sponge biomass (Vacelet 1975, Wilkinson 1978b, Willenz and Hartman 1989). These species were therefore termed high-microbial abundance sponges or bacteriosponges (Hentschel et al. 2003).

Bacteriosponges contain microbial communities that are unusually diverse in morphology as well as in phylogeny. At least seven different morphotypes of sponge associates were described based predominantly on different membrane structures (Vacelet 1975, Wilkinson 1978b, Friedrich et al. 1999, Fieseler et al. 2004). The three most dominant ones are characterized by several additional membranes, by a copious, irregular slime layer, and by a putative nuclear membrane and they were consistently found in sponges over time and space (Fig. 3D, E). It was proposed that these additional structures mask the microorganisms and thereby prevent their detection and digestion by sponge cells (Friedrich et al. 1999, Wilkinson et al. 1984). The different morphotypes are found equally scattered in the mesohyl indicating a well mixed microbial community rather than niche adaptation by different microbes within the sponge. The phylogenetic diversity of the associated microbial community was determined using 16S rDNA as molecular marker (Hentschel et al. 2002, Hill et al. 2006, Olson and McCarthy 2005, Schirmer et al. 2005, Taylor et al. 2005, Webster et al. 2001a). To date, phylotypes within nine eubacterial phyla are identified as members of the sponge associated community (Fig. 4). Many phylotypes fell within the Proteobacteria (Alpha, Beta, Gamma, and Delta Subdivisions) and Chloroflexi. A moderate number of phylotypes are affiliated with Acidobacteria and Actinobacteria whereas Bacteroidetes, Cyanobacteria, Gemmatimonadetes and Nitrospira contain one or two phylotypes. The candidate phylum Poribacteria is special in this context as it to date exclusively contains sequences from marine sponges (Fieseler et al. 2004). The total number of different bacterial strains within the sponge associated community is unknown but rough estimates based on 16S rDNA libraries suggest that more than 50 but probably less than 100 bacterial species can be expected (S. Schmitt, pers. observation). This microbial diversity by far exceeds other invertebrate-microbe associations that usually consist of one or few microbes (Schmitt et al. in press). Because the bacterial composition in sponges is notably different from seawater a sponge-specific microbial community was proposed that seems specifically adapted to sponges (Hentschel et al. 2002). None of these specific bacteria has been cultivated to date and

therefore the phenotypic, physiological, and biochemical properties of sponge specific bacteria are almost unknown.

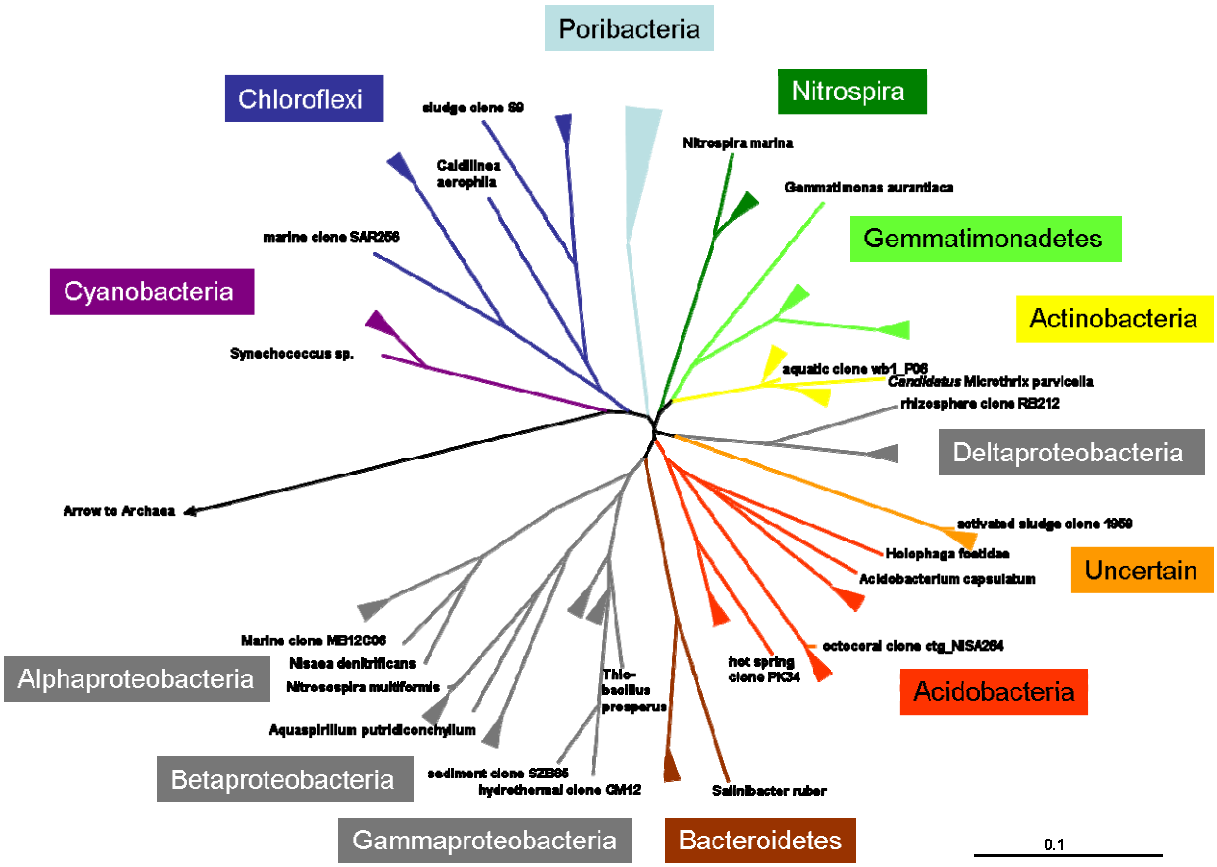

Fig. 4: Phylogenetic tree representing sponge specific clusters (triangles) within at least nine eubacterial phyla. Scale bar indicates 10% sequence divergence.

Another interesting aspect is that the specific microbial community is associated with many different host species. There seems to be no correlation between microbial abundances and sponge taxonomy as the host sponges belong to different orders although at least one sponge order (Verongida) appears to contain exclusively bacteriosponges. Furthermore, these host species are not restricted to one geographical area but exist in tropical as well as in temperate regions and inhabit coral reefs, mangroves, and even the deep sea (Hentschel et al. 2002, Hill et al. 2006, Olson et al. 2005, Taylor et al. 2005, Webster et al. 2001a). A remarkable example is the cluster of exclusively sponge derived sequences that are affiliated with the cyanobacteria *Synechococcus* and *Prochlorococcus* (Steindler et al. 2005). This cluster

contains sequences from 16 different sponge species from a wide range of geographic locations (Australia, Red Sea, Mediterranean Sea, Caribbean Sea). To date, it is not well understood why these different host species are associated with the same specific microbial community but sponge morphology, chemistry and ecological factors may be important.

The stability of the bacteriosponge associated microbial community was investigated in several recent studies. Temporal as well as intraspecies variability in the microbial community was found to be minimal (Friedrich et al. 2001, Taylor et al. 2004). Spatial variability also seems to be low as indicated by the wide geographic distribution of bacteriosponges (Hentschel et al. 2002, Taylor et al. 2005), although sometimes environmental factors such as light and temperature may influence the microbial community (Taylor et al. 2005). Experimental influences such as exposure to sub lethal copper concentrations resulted in decreased microbial numbers and diversity (Webster et al. 2001b) whereas exposure to antibiotics, starvation under laboratory conditions in an aquarium or the *in situ* transplantation from the natural location to shallower places revealed almost no changes in the microbial community (Friedrich et al. 2001, Thoms et al. 2003). The stability over time and space and the specificity of the association point to a closely adapted system.

To date, it is still unclear whether the nature of the demosponge - microbe association is commensal (living together without detriment or benefit) or mutualistic (living together with reciprocal benefit). Several beneficial functions of the associated microbes have been proposed including provision of nutrients through photosynthesis, nitrification, and N_2-fixation (Vacelet 1971, Wilkinson and Fay 1979), removal of waste products (Wilkinson 1978a), production of secondary metabolites as chemical defences against predators and biofouling (Proksch et al. 2002, Blunt et al. 2006), protection from UV radiation (Wilkinson 1980) and stabilization of the sponge skeleton (Wilkinson et al. 1981). Considering that the sponge is a suitable habitat for microorganisms, these data indicate a mutualistic relationship with benefits for both partners. However, none of these functions has been proven so far, although the involvement in the sponge carbon and particularly nitrogen metabolism appears most likely (Bayer et al. submitted). Nevertheless, because of the lack of unambiguous evidence the association is regarded to be commensal in this Ph.D. thesis.

III. Evidence of microorganisms in reproductive stages and cospeciation

Vertical transmission of microorganisms has been proposed as a mechanism by which apparently permanent, stable and specific sponge – microbe associations may be established and maintained. Levi and Porte (1962) were the first to use electron microscopy to document the presence of microorganisms in the larvae of the sponge *Oscarella lobularis*. Subsequent electron microscopy studies revealed the presence of microorganisms in oocytes, embryos and larvae of many oviparous as well as viviparous species within all three poriferan classes (Table 1). However, only in a few cases microbial incorporation and the whole process of vertical transmission from one generation to the next could be shown. For example, Ereskovsky et al. (2005) described the transmission of a small spiral bacterium from the oocyte to metamorphosis of the larva in the viviparous sponge *Halisarca dujardini*. The bacterium was taken up by the oocyte via endocytosis. During embryogenesis the bacterium was located inside and between blastomeres until it was present in the central part of the larva as well as between the ciliated cells at the larval epithelium. Finally, during metamorphosis the bacterium was distributed in the extracellular space at different parts of the rhagon. Because no other microbial morphotypes were detected in *H. dujardini* this association might represent a specific relationship between this species and the spiral bacterium. Similarly, the deep-sea sponge *Cladorhiza methanophila* is associated with only two different bacterial morphotyes that were also detected by electron microscopy within flattened cells at the surface of embryos (Vacelet et al. 1995, 1996). Accordingly, direct transmission of the symbionts between generations was proposed by the author.

The cyanobacterium *Oscillatoria spongeliae* might have evolved host specificity with dictyoceratid sponges of the genus *Dysidea* and cospeciated with its host as suggested by congruent phylogenies (Thacker and Starnes 2003). Cospeciation can only be achieved when the symbiont is strictly vertically transmitted and this seems to be the case for the *Dysidea- O. spongeliae* association. However, when the dataset was subsequently extended by additional dictyoceratid species from another location it appeared that at least one host switching event took place (Ridley et al. 2005a) indicating that also horizontal transfer of *O. spongeliae* strains must have occurred. Hence, the authors stated that the speciation of *O. spongeliae* strains and its dictyoceratid hosts is more complex than currently thought.

Table 1: Compilation of species with microbes in reproductive stages detected by TEM

	Order/ Species	Bacteriosponge	Reference
	Species with oviparous mode of reproduction		
DEM[1]	Astrophorida		
	Erylus discophorus	yes	Sciscioli et al. 1989
	Geodia cydonium	yes	Sciscioli et al. 1994
	Stelletta grubii	yes	Sciscioli et al. 1991
	Chondrosida		
	Chondrilla spp.	yes	Gaino 1980, Usher et al. 2001
	Chondrosia reniformis	yes	Levi and Levi 1976
	Hadromerida		
	Tethya spp.	no	Gaino and Sara 1994
	Poecilosclerida		
	Hemectyon ferox	n.d.	Reiswig 1976
	Verongida		
	Aplysina aerophoba	yes	Gallissian and Vacelet 1976
	Species with viviparous mode of reproduction		
DEM[1]	Dictyoceratida		
	Hippospongia lachne	yes	Kaye 1991, Kaye and Reiswig 1991
	Ircinia oros	yes	Ereskovsky and Tokina 2004
	Spongia spp.	yes	Kaye 1991, Kaye and Reiswig 1991
	Hadromerida		
	Alectona spp.	yes	Garrone 1974, Vacelet 1999
	Halichondrida		
	Swenzea zeai	yes	Rützler et al. 2003
	Halisarcida		
	Halisarca dujardini	no	Ereskovsky et al. 2005
	Haplosclerdia		
	Haliclona tubifera	n.d.	Woollacott 1993
	Homoscleromorpha		
	Oscarella spp.	yes	Levi and Porte 1962, Ereskovsky and Boury-Esnault 2002
	Poecilosclerida		
	Cladorhiza sp.	no	Vacelet et al. 1995, 1996
	Hamigera hamigera	yes	Boury-Esnault 1976
	Verticillittida		
	Vaceletia crypta	yes	Vacelet 1979
CAL[1]	Leucosolenida		
	Grantia compressa	no	Lufty 1957, Gallissian 1983
	Scypha ciliata	no	Franzen 1988
HEX[1]	Lyssacinosida		
	Oopsacas minuta	no	Boury-Esnault et al. 1999

n.d.: not determined

[1] Sponge classes CAL: Calcarea, DEM: Demospongiae, HEX: Hexactinellida

Gallissian and Vacelet (1976) used electron microscopy to described vertical transmission of the morphologically complex microbial community present in adult *Aplysina* spp.. They could show that maternal mesohyl microorganisms are endocytosed by early oocytes via pseudopodia and are translocated in vesicles to the peripheral region of the oocytes. At the end of oocyte development the number of microorganisms increased dramatically due to microbial cell division. None of these microbes showed signs of digestion and the authors concluded that the microbes do not have a trophic function but are transmitted to the next generation (Gallissian and Vacelet 1976). Kaye (1991) also reported on vertical transmission of a complex microbial community in the viviparous species *Hippospongia lachne* and *Spongia* spp.. In contrast to *Aplysina* sponges, in these species oocytes and zygotes were devoid of microorganisms. At the embryonic stage, umbilici were established to extracellularly transfer maternal mesohyl microorganisms to the offspring where the microbes were located between blastomeres. Finally, the microorganisms were observed intercellularly in larvae and post-larvae (Kaye and Reiswig 1991). *Aplysina* spp., *Hippospongia* sp., and *Spongia* spp. as well as *Chondrilla* spp. and *Chondrosia* sp. (Table 1) all belong to the group of demosponges that are associated with a specific microbial community. Despite the varying time and mechanism of microbial corporation electron microscopy of the respective reproductive stages gave a first morphological indication that the association is maintained by vertical transmission. At the beginning of this Ph.D. thesis the microorganisms within sponge reproductive stages were documented only by electron microscopy and the phylogenetic identity of the microbial community within sponge reproductive stages was entirely unknown.

IV. Aims of the thesis

Bacteriosponges are associated with morphologically and phylogenetically complex microbial communities. Electronmicroscopy data indicate the presence of microorganisms in sponge reproductive stages of certain species. However, their phylogenetic identity was unknown at the beginning of this PD. D. thesis.

The first aim of this Ph.D. thesis was to investigate cospeciation of *Aplysina* sponges and sponge specific cyanobacterial phylotypes. In a first step the genealogical relationships between eight *Aplysina* species from the Mediterranean and Caribbean Sea were resolved using three different molecular markers (chapter 2). In a second step this host phylogeny was

compared with the phylogeny of *Synechococcus*-related cyanobacteria in chapter 7 (general discussion) and the hypothesis of cospeciation is discussed.

The second aim of this Ph.D. thesis was to investigate vertical transmission of the sponge specific microbial community. First, an electron microscopy survey was performed on each of five bacteriosponges and non-bacteriosponges (chapter 3). The adult and respective reproductive stages were inspected for the presence of microorganisms to establish general patterns of vertical transmission.

Based on the results of chapter 3, the viviparous sponge *Ircinia felix* was chosen for a detailed examination of vertical transmission. *In situ* settlement experiments were performed and denaturing gradient gel electrophoresis (DGGE) was applied to compare microbial profiles between adult, larvae and juveniles, between larvae released by the same adult specimen, and between larvae released by different specimens. For the first time phylogenetic sequence information about microorganisms in sponge reproductive stages is provided. The results and consequences of vertical transmission in *I. felix* are reported in chapters 3, 4, and 5.

In chapter 6, a comprehensive sequence analysis including over 200 sequences from sponge reproductive stages was performed to investigate whether vertical transmission of specific phylotypes in sponges can be generalized.

Finally, chapter 7 provides a general discussion of cospeciation and vertical microbial transmission in bacteriosponges. Consequences and implications for the ecology and evolution of the association are discussed and future perspectives are given.

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Chapter Two

ITS-2 and 18S rRNA gene phylogeny of Aplysinidae (Verongida, Demospongiae)

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I. Abstract

18S ribosomal DNA and internal transcribed spacer 2 (ITS-2) full length sequences, each of which was sequenced three times, were used to construct phylogenetic trees with alignments based on secondary structures, in order to elucidate genealogical relationships within the Aplysinidae (Verongida). The first poriferan ITS-2 secondary structures are reported. Altogether eleven *Aplysina* sponges and three additional sponges (*Verongula gigantea, Aiolochroia crassa, Smenospongia aurea*) from tropical and subtropical oceans were analyzed. Based on these molecular studies, *S. aurea* that is currently affiliated with the Dictyoceratida should be reclassified to the Verongida. *Aplysina* appears as monophyletic. A soft form of *Aplysina lacunosa* was separated from other *Aplysina* and stands at a basal position in both 18S and ITS-2 trees. Based on ITS-2 sequence information, the *Aplysina* sponges could be distinguished into a single Caribbean-Eastern Pacific cluster and a Mediterranean cluster. The species concept for *Aplysina* sponges as well as a phylogenetic history with a possibly Tethyan origin are discussed.

II. Introduction

Sponges represent the base of metazoan evolution with a fossil record dating back to the Precambrian times (Bergquist 1978; Li et al. 1998). Today an estimated 15,000 sponge species have been described but the true diversity is probably still much higher (Hooper and Van Soest 2002). Traditionally, the sponges (Porifera) are divided into three classes, the Hexactinellida (glass sponges), the Calcarea (calcareous sponges) and the Demospongiae, the latter of which contains more than 85% of the species living today. Sponges are diploblast metazoans that lack true tissues or organs. In spite of their basal position in the eukaryotic tree, genome sequencing has revealed genes that are highly homologous to vertebrate gene sequences (Böhm et al. 2000; Müller et al. 2002). The key diagnostic characters used for sponge taxonomy are the spicules and spongin fibres allowing genus level identification for many poriferan groups (Hooper and Van Soest 2002; Zea 1987). However, because of the paucity of useful morphological features, a precise taxonomical classification of the Porifera continues to be problematic. While a need for molecular phylogeny of the Porifera has long

been recognized, it has only partially been met with success (see Borchiellini et al. 2000 and van Oppen et al. 2002 for reviews).

Aplysinidae (Verongida Bergquist, 1978) have received attention because of their widespread abundances, notably in the Caribbean and the Mediterranean, their diverse chemistry and because of their association with large amounts of microorganisms (Vacelet 1975; Hentschel et al. 2003). Sponges of the Aplysinidae (see review in Bergquist and Cook 2002a; b) are usually sulfur-yellow in color but can be tinged towards green or red due to the presence of copious amounts of cyanobacteria in the ectosomal layer. Their shape varies from tall tubular vases (*Aplysina archeri*) and spreading colonies with multiple chimneys (*A. cavernicola*) to creeping ropes (*A. cauliformis*). The mesohyl consistency is homogenous, fleshy and deformable due to the presence of large amounts of collagen. Spongin fibers are rather tough, made up of a laminated, golden bark and a granular, dark pith. Mineral spicules are lacking in these keratose sponges. The choanocyte chambers are diplodal and small. The reproduction is oviparous but documentation is scarce (Gallissian and Vacelet 1976; Tsurumi and Reiswig 1997). More than 100 different bromotyrosine-derived metabolites have been identified and occur in all verongid species investigated so far (Carney and Rinehart 1995). Another characteristic of this sponge group is the color change from yellow to blue and black upon injury or exposure to air due to the oxidation of pigments (Cimino et al. 1984). The Aplysinidae contain two genera, the *Aplysina* and *Verongula* while a third one, *Aiolochroia* (incertae sedis) has tentatively been affiliated with this family (Bergquist and Cook 2002b). The aim of this study was to construct phylogenetic trees based on rRNA gene sequence information for this important and abundant marine sponge taxon.

III. Material and Methods

Sponge collection

The Mediterranean sponges *Aplysina aerophoba* and *A. cavernicola* were collected by scuba diving offshore Banyuls-sur-Mer, France at 5 - 15 m depth (42°29´N; 03°08´E) in May 2000 and Elba, Italy at depth below 40 m (42°43´N; 10°08´E) in May 2001, respectively. The Caribbean sponges *A. archeri*, *A. insularis*, *A. cauliformis* (thick and thin morphotype), *A. lacunosa* (soft and hard morphotype), were collected by scuba diving at depths of 5 - 20 m offshore Little San Salvador Island, Bahamas in July 2000 (24°32´N; 75°55´W). The sponges

A. fistularis, *Verongula gigantea*, *Aiolochroia crassa* and *Smenospongia aurea* were also sampled offshore Little San Salvador Island at GPS position 25°20´N; 76°53´W. The mangrove sponge *A. fulva* was collected offshore Sweetings Cay, Bahamas, at 2 m depth (26°33´N; 77°53´W). An Eastern Pacific sample of *Aplysina fistularis* was taken offshore Scripps Institution of Oceanography, San Diego, California, USA (32°51´N; 117°15´W) (Table 1). Specimens were brought to the surface in separate plastic bags; small pieces of tissue were removed using an EtOH-sterilized cork borer, frozen in liquid nitrogen and stored at – 80°C until use. Voucher specimens are available upon request.

Table 1: Classical taxonomy of Aplysinidae (Verongida, Demospongiae) based on morphology (Bergquist and Cook 2002a; Zea 1987) and GenBank accession numbers of 18S rDNA and ITS-2 sequences.

Order	Family	Genus	Species	18S rDNA[a, b]	ITS-2[a]
Verongida	Aplysinidae	*Aplysina*	*A. aerophoba* Schmidt, 1862	AY591799	AY591786
			A. cavernicola (Vacelet, 1959)	AY591800	AY591787
			A. archeri (Higgin, 1875)	AY591801	AY591788
			A. lacunosa (Pallas, 1766) (soft)[c]	AY591803	AY591796
			A. lacunosa (Pallas, 1766) (hard)[c]	AY591802	AY591795
			A. cauliformis (Carter, 1882) (thick)[c]	n.d.	AY591790
			A. cauliformis (Carter, 1882) (thin)[c]	n.d.	AY591789
			A. insularis (Duch. and Mich. 1864)[d]	n.d.	AY591794
			A. fistularis (Pallas, 1766) (Bahamas)[d]	n.d.	AY591791
			A. fistularis (Pallas, 1766) (California)[e]	n.d.	AY591792
			A. fulva (Pallas, 1766)	n.d.	AY591793
		Verongula	*V. gigantea* (Hyatt, 1875)[f]	AY591804	AY591797
		Aiolochroia[g]	*A. crassa* (Hyatt, 1875)	AY591805	AY591798[h]
Dictyoceratida	Thorectidae	*Smenospongia*	*S. aurea* (Hyatt, 1875)[i]	AY591806	

n.d.: not determined. A wider taxon sampling was performed for the ITS-2 phylogenetic analysis.

[a] GenBank accession numbers.

[b] Accession Numbers of additional sequences in the 18S tree obtained from GenBank: *Acanthochaetetes wellsi*: AF084237; *Aplysilla sulfurea*: AF246618; *Axinella polypoides*: APU43190; *Clathria reinwardti*: AF084238; *Clypeatula cooperensis*: AF140354; *Dysidea avara*: AF456326; *Ephydatia muelleri*: AF121110; *Eunapius fragilis*: AF121111; *Hippospongia communis*: AF246616; *Microciona prolifera*: L10825; *Mycale fibrexilis*: AF100946; *Negombata corticata*: AF084239; *Oopsacas minuta*: AF207844; *Plakortis sp.*: AF100948; *Pleraplysilla spinifera*: AF246617; *Rhabdocalyptus dawsoni*: AF100949; *Spongilla lacustris*: AF121112; *Suberites ficus*: AF100947; *Tetilla japonica*: D15067.

[c] Two sympatric morphotypes.

[d] Caribbean *A. fistularis* and *A. insularis* may be ecophenotypes or developmental stages of the same species (see Wiedenmayer 1977 and Van Soest 1978).

[e] Possibly synonymous to *A. thiona* (de Laubenfels, 1930).

[f] Thin-walled, bowl-shaped form [compared to *V. reiswigi* which is the sympatric thick-walled, jug-shaped form, Alcolado, 1984]; we follow Wiedenmayer (1977) rather than Bergquist and Cook (2002b) in preferring *V. gigantea* over *V. praetexta* as the valid name for this species. Whereas *V. gigantea* forms a single bowl, *V. praetexta* forms a cluster of tubes arising from a spreading base.

[g] Incertae sedis.

[h] The ITS-2 sequence of *Aiolochroia* was excluded from the tree, because it was too variable and therefore *Verongula* was preferred as outgroup.

i Synonymous to *S. cerebriformis* (Duchassaing and Michelotti, 1864).

DNA extraction, amplification and cloning

DNA extraction from liquid nitrogen-frozen samples was performed using the Fast DNA Spin kit for soil (Q-Biogene, Heidelberg, Germany) in accordance with the manufacturer´s instructions. Two different strategies were used for full length 18S rDNA amplification: (1) For *Aplysina aerophoba*, *A. archeri*, *Verongula gigantea*, *Aiolochroia crassa* and *Smenospongia aurea,* the 18S rDNA was amplified with primer A (5'-AACCTGGTTGATCCTGCCAGT-3') and primer B (5'-TGATCCTTCTGCAGGTTCACC-TAC-3') described by Medlin (Medlin et al. 1988). Additionally, the internal 1300 bp long fragment was amplified with the primers 18Sf20 (5'-TGGTACGGTAGTGGCCTACCATG-G-3') and 18Sr21 (5'-ACGGGCGGTGTGTACAAAGGGCAG-3') (McInerney et al. 1999). (2) The 18S rRNA gene of *A. cavernicola*, *A. lacunosa* (hard) and *A. lacunosa* (soft) was amplified in two overlapping fragments using the primer pairs A/1r (5'-TGGTGCCCTTCCGTCAATTCCT-3' and 2f (5'-CTGGTGCCAGCAGCCGCGG-3')/B (Primers A/B, Medlin et al. 1988; primers 1r/2f modified after Kelly-Borges and Pomponi 1994) as described by Peterson and Addis (2000). Amplification of the ITS-2 region was performed with the primers RA2 (5'-GTCCCTGCCCTTTGTACACA-3') and ITS2.2 (5'-CCTGGTTAGTTTCTTTTCCTCCGC-3') (Wörheide et al. 2002a; b). PCR cycling conditions were as follows: initial denaturation (2 min at 95°C) followed by 30 cycles of denaturation (1 min at 95°C), primer annealing (1 min at temperatures adjusted for each primer set, see below) and primer extension (1.5 min at 72°C) and a final extension step (10 min at 72°C). Annealing temperatures for the primer sets were: Primer A/ Primer B: 60°C; 18Sf20/ 18Sr21: 65°C; Primer A/ 1r: 55°C; Primer 2f/ Primer B: 70°C, Primer RA2/ ITS.2: 67°C. PCR products were ligated into the pGEM-T-easy vector (Promega) and transformed by electroporation into competent *E. coli* XL 1-Blue. Plasmid DNA was isolated by standard miniprep procedures and the correct insert size was verified by using agarose gel electrophoresis following restriction digestion.

DNA sequencing

Sequencing was performed on a LiCor 4200 automated sequencer (LiCor, Inc., Lincoln, Nebr.) and on an ABI 377XL automated sequencer (Applied Biosystems). Each 18S rDNA or ITS gene fragment was sequenced three times on both strands. The sequences were aligned automatically using the ContigExpress tool in Vector NTI suite 6.0 (InforMax, Inc) and were refined by eye. The final sequences, thereafter referred to as 'consensus sequences', were

deposited in GenBank/EMBL/DDBJ under the accession numbers AY591786 - AY591806 (Table 1).

Alignments and phylogenetic analyses

All newly obtained 18S rRNA gene sequences were aligned with other sponge sequences (obtained by GenBank [http://www.ncbi.nlm.nih.gov/]) according to conserved patterns of the secondary structure model of the 18S rRNA (Gutell et al. 1985). ITS-2 sequences were aligned according to conserved patterns of the secondary structure model of the ITS-2 as derived in this study (Fig. 1). Alignments and direct comparison of sequences were carried out manually with the Windows-based multicolor sequence alignment editor of Hepperle (2002) and in case of the ITS-2 region, additionally with the help of MARNA (Siebert and Backofen 2003). The aligned RNA sequences were of similar lengths, with the exception of few species-specific insertions or variant regions. Whenever proper alignment was not possible, the corresponding sequence parts were excluded from further analyses. The ITS-2 and 18S alignments, displayed by CHROMA (Goodstadt and Ponting 2001) are available from the authors on request. Phylogenetic analyses of aligned sequences were conducted using PAUP* version 4.0b10 win32 (Swofford 2002). For parsimony analyses (MP) (Camin and Sokal 1965) heuristic searches with 10 random taxon addition replicates and tree bisection-reconnection (TBR) swapping were applied. MulTrees and Collapse options of PAUP were used and character changes were interpreted with ACCTRAN (accelerated transformation) optimization. Characters were equally weighted and coded as unordered; gaps were treated as missing data. Bootstrap support (Felsenstein 1985) was estimated based on 100 pseudo-replicates. Minimum evolution (ME) trees were generated using maximum likelihood genetic distances, heuristic searches, TBR swapping, and a starting tree obtained via neighbor-joining (Saitou and Nei 1987). Bootstrap support also was estimated based on 100 pseudo-replicates. For maximum likelihood analysis (ML) (Felsenstein 1981) again heuristic searches and a 100-fold bootstrap analysis were applied. NNI (nearest neighbour interchange) was used as branch swapping algorithm. A puzzle analysis (Strimmer and von Haeseler 1996) as implemented in PAUP, was performed with 10.000 quartet puzzling steps (set criterion = likelihood). All trees were rooted by a user specified outgroup and displayed by using the TreeView program (Page 1996)

For distance and ML analyses, which are dependent on a particular specified model of nucleotide substitution, the program Modeltest (Posada and Crandall 1998), was used to

survey 56 possible models of DNA evolution to identify the model that best fits the rDNA datasets. Likelihood settings from the best-fit model were determined using the AIC criterion (Akaike Information Criterion). For the 18S phylogenies as shown here, the TIM+I+G model (Transition model) was chosen, with estimates of nucleotide frequencies A = 0.25110, C = 0.20020, G = 0.27430, T = 0.27440; a rate matrix with six different substitution types, assuming a heterogeneous rate of substitutions with a gamma distribution of variable sites (number of rate categories = 4, shape parameter [alpha] = 0.6228); and the proportion of invariant sites that are unable to accept substitutions (pinvar) = 0.5506.

For the ITS-2 phylogenies as shown here, the TrN+I model was chosen with estimates of nucleotide frequencies A = 0.17520, C = 0.30380, G = 0.31100, T = 0.21000; and a rate matrix with six different substitution types; and the proportion of invariant sites that are unable to accept substitutions (pinvar) = 0.3090.

Additionally a Bayesian approach to tree reconstruction as implemented in the MrBayes program by Huelsenbeck and Ronquist (2001) was based on a general time reversible (GTR) substitution model (Lanave et al. 1984; Rodriguez et al. 1990; Tavaré 1986) with a gamma rate distribution estimated from the data set. For 18S and ITS-2 data, in each case, starting from random trees, four Markov chains (one cold, three heated) run in parallel to sample trees using the Markov Chain Monte Carlo (MCMC) principle. After the burn-in phase every 100-th sample out of 1.000.000 generations was considered, and results were compared among the four chains in order to confirm, that stationarity has been reached. Finally, 50% majority rule consensus trees were constructed.

ITS-2 secondary structure prediction

The 5’ and 3’ ends of the ITSs were determined in accordance with GenBank designations of other sponge sequences. In general, secondary structure prediction was performed as described in Mai and Coleman (1997). Briefly, ITS-2 secondary structures were derived by using RNAstructure (Mathews et al. 1999). Common structural elements were initially recognized by screening for thermodynamically optimal and suboptimal secondary structures. Foldings were conducted at 37°C. When sequences are folded, lower-case bases were used as constraints for bases that were not allowed to base pair. Structures were displayed using loopDloop (Gilbert 1996). Compensatory base changes (CBCs) were counted using RNAforester (Höchsmann et al. 2003).

IV. Results

Methodological considerations

The sequence variability due to possible methodological errors (i.e., Taq-infidelity, sequencing ambiguities) was assessed. Three independent sequencing reactions of two clones each were compared and the variability was found to be between 2.4 - 4.4%. In order to minimize sequencing errors, each rDNA region was routinely sequenced three times on both strands and the visually corrected sequence (hereafter termed 'consensus sequence') was used for subsequent phylogenetic analysis. With this sequencing strategy, the ambiguities were reduced to 0.30 - 0.68%. In order to screen for intragenomic polymorphism (IGP) due to presence of multiple ribosomal gene copies, the ITS-2 sequence variability between eight clones from a single *A. aerophoba* specimen was compared. Six of the eight clones were identical while two clones showed three variable sites each. In order to screen for intraspecies polymorphism, the ITS-2 region from four specimens of *A. aerophoba* was sequenced and was found to be 100% identical between all four specimens. Therefore the low extent of intragenomic variation can probably be neglected for phylogenetic analysis.

18S rRNA phylogeny

Based on 18S rRNA gene sequence data and with respect to the outgroup (Hexactinellida), the Demospongiae appeared as monophyletic (Fig. 1). Within the Demospongiae, the 18S rRNA gene tree largely reflects previous trees based on complete 18S rRNA gene sequences (Peterson and Addis 2000; Borchiellini et al. 2001). For the orders Poecilosclerida and Haplosclerida the molecular trees consistently support their taxonomic placement following Hooper and Van Soest (2002). With respect to the orders Hadromerida and Dendroceratida, the molecular trees consistently differ from their taxonomic placements following morphological classification (Hooper and Van Soest 2002). Special interest was placed on the resolution of the Aplysinidae. *Smenospongia* that is currently affiliated with the Thorectidae (Dictyoceratida) due to the presence of the shared metabolite, Aplysinopsin, with other thorectid sponges (Cook and Bergquist 2002) was placed as the sister group to *Verongula* and *Aplysina*. All three groups sister to *Aiolochroia*. The genus *Aplysina* appeared as monophyletic but was supported up to 75% only when *Aplysina lacunosa* (soft) was excluded from the analysis. *Aplysina cavernicola* sisters to *Aplysina aerophoba,* and both species together sister to *Aplysina lacunosa* (hard). The latter three taxa sister to *Aplysina archeri.*

Resolution within *Aplysina* was low, i.e., especially maximum parsimony and distance analyses yielded multifurcations and low bootstrap support values.

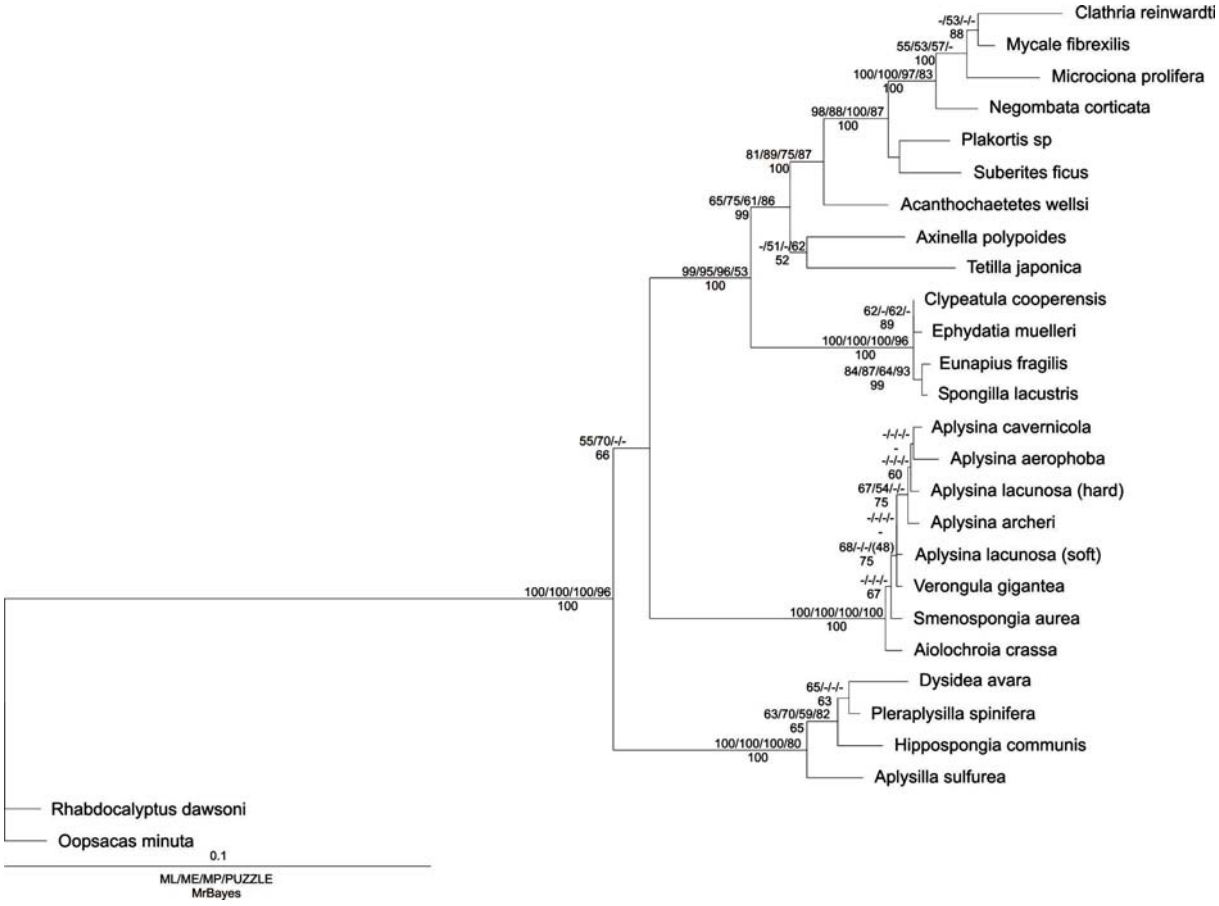

Fig. 1: Maximum likelihood tree as derived from PAUP analyses of 18S rRNA gene sequences using TIM + I + G. –ln likelihood = 6866.53616. The length of the alignment was 1857 characters, of which 324 were parsimony informative; 1448 characters are constant, and 85 variable characters are parsimony uninformative. Tree length = 809; tree of the same length, n = 37; consistency index (CI) = 0.6489; homoplasy index (HI) = 0.3511; CI excluding uninformative characters = 0.6034; HI excluding uninformative sites = 0.3966; retention index (RI) = 0.5430. Numbers at the nodes indicate bootstrap values as calculated by ML, ME, MP, and PUZZLE support values (>50) (above branches) as well as posterior probabilities as calculated by MrBayes (below branches).

ITS-2 phylogeny and secondary structure

Using a wider taxon sampling with *Verongula* as outgroup, *Aplysina* again appeared as monophyletic (Fig. 2). Consistent with the 18S rRNA gene tree, *A. lacunosa* (soft) clustered at a basal position, followed by (i) *Aplysina cavernicola* plus *Aplysina aerophoba* and (ii) a large cluster including all other *Aplysina* species as determined in this study. No further resolution could be obtained as there are only 1- 8 base differences within the *A. archeri – A. fistularis* group. In maximum likelihood analyses *A. lacunosa* (soft) showed a week affinity to *A. cavernicola* plus *A. aerophoba*, supporting its morphological differences to *A. lacunosa* (hard) and a different phylogenetic placement. The ITS-2 secondary structures preserve the familiar four helix domain known e.g. from green algae and flowering plants (Mai and Coleman 1997), dinoflagellates (Gottschling and Plötner 2004), diatoms (Wolf 2004) or Metazoa (e.g. Coleman and Vacquier 2002, Joseph et al. 1999) also including the universally conserved U-U mismatch in helix II (Fig. 3). Further statistics on the secondary structures are given in Table 2.

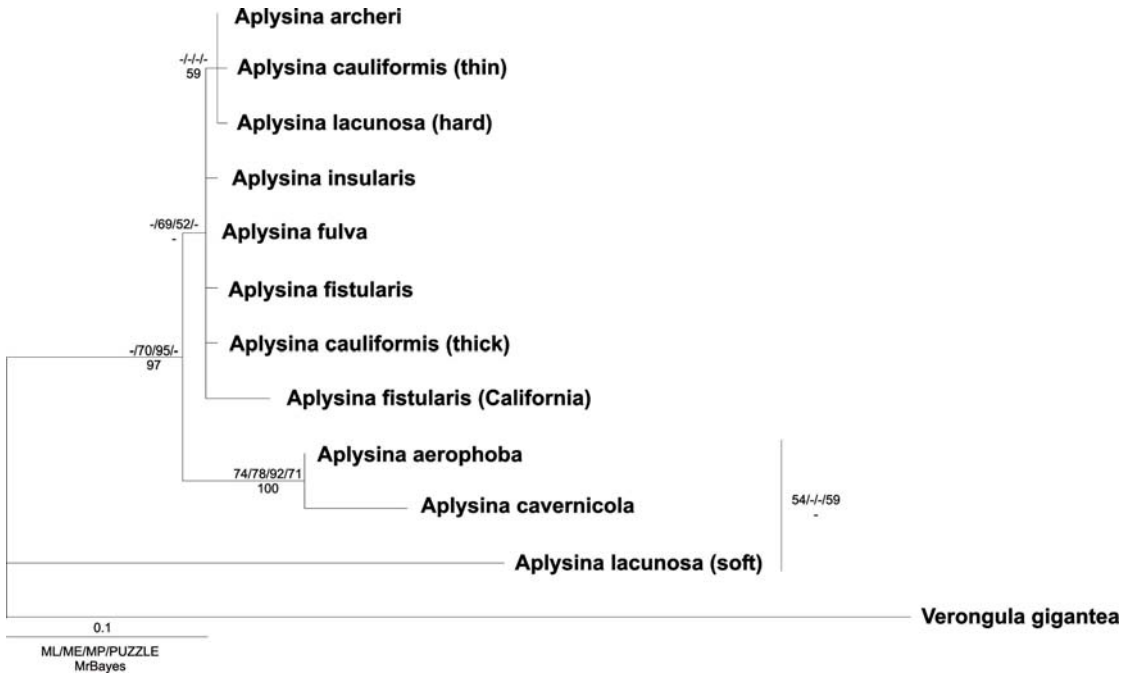

Fig. 2: Maximum likelihood tree as derived from PAUP analyses of ITS-2 sequences using TrN + I. –ln likelihood = 838.79841. The length of the alignment was 260 characters, of which 26 were parsimony informative. 161 characters are constant; 73 variable characters are parsimony uninformative. Tree length = 125; trees of the same length, n = 5; consistency index (CI) = 0.9440; homoplasy index (HI) = 0.0560; CI excluding uninformative characters = 0.8250; HI excluding uninformative sites = 0.1750; retention index (RI) = 0.7941; rescaled consistency index (RC) = 0.7496. Numbers at the nodes indicate bootstrap values as calculated by ML, ME, MP, and PUZZLE support values (>50) (above branches) as well as posterior probabilities as calculated by MrBayes (below branches).

V. Discussion

The conserved sequence of 18S rRNA genes are commonly used for elucidating relationships between higher taxonomic units (Adams et al. 1999; Borchiellini et al. 2001; Kelly-Borges et al. 1991; Kelly-Borges and Pomponi 1994; McInerney et al. 1999) while the more variable 28S rDNA sequences are employed for resolving relationships from the order to the genus level (Alvarez et al. 2000; Chombard et al. 1997; 1998; McInerney et al. 1999). The ITS-2 spacer was long considered too variable for phylogenetic analysis (Chombard et al. 1997; 1998) but was useful for species level resolution of *Dysidea* and *Axinella* sponges, respectively (Thacker and Starnes 2003, Lopez et al. 2002). Though coding genes like mitochondrial cytochrome oxidase subunit 1 (COI) or protein kinase C (PKC) are considered appropriate to elucidate relationships between species due to a highly variable DNA sequence they either failed at this level (Schröder et al. 2003) or were used for other purposes, e.g. resolving evolutionary relationships of Metazoa (Schütze et al. 1999) or of poriferan classes (Kruse et al. 1998). A significant improvement was made by taking the respective rRNA secondary structures into account. Because the secondary structure is more conserved than the primary structure, it allows a proper alignment even when sequences are highly diverse. Therefore, the use of secondary structure is not only critical for the alignment but also for the phylogeny derived from it. Using the secondary structure for alignment, the resolution power of the ITS-2 sequence could be extended from orders to subspecies and even to spatially isolated populations (Coleman 2003). To our knowledge, only one molecular study has successfully used secondary structure information for poriferan 28S alignment in order to investigate the phylogenetic relationships of the Axinellidae (Alvarez et al. 2000).

The results of this study are largely congruent with previous phylogenetic trees using full length 18S rRNA gene sequences (Borchiellini et al. 2001; Peterson and Addis 2000) in that most higher level poriferan relationships are resolved with good bootstrap support (Fig. 2). However the molecular data differ noticeably from the currently valid taxonomy based mainly on morphological characteristics (Hooper and Van Soest 2002) in that the Hadromerida and Dendroceratida appear as para- or polyphyletic. So far only four taxa referred as orders are represented by two or more full length 18S sequences (Hadromerida, Dendroceratida, Poecilosclerida, Haplosclerida). Accordingly, there is a continued need for taxonomic re-evaluation of sponges as more complete 18S sequences become available.

The Aplysinidae which are represented by complete sequences of all three genera (*Aplysina*, *Verongula* and *Aiolochroia* as incertae sedis) appear as paraphyletic because of the position of *Smenospongia aurea* (Dictyoceratida). Interestingly this species shows features characteristic of verongid sponges, e.g. a typical color change by contact with air or upon death. On the other hand, *S. aurea* is currently grouped with the Dictyoceratida, because of the presence of tryptophane-derived secondary metabolites which have not been observed in verongid sponges. Nevertheless molecular data clearly confirm the affiliation of *S. aurea* with the Aplysinidae and therefore a reclassification of this species with the Aplysinidae is suggested. The family placement of the genus *Aiolochroia* is uncertain. Current knowledge of skeleton structure and histological characteristics as well as the geographical distribution of this taxon points to a status within the Aplysinidae. However, because no sequences of other verongid families were included here, the exact position of *Aiolochroia* awaits further confirmation.

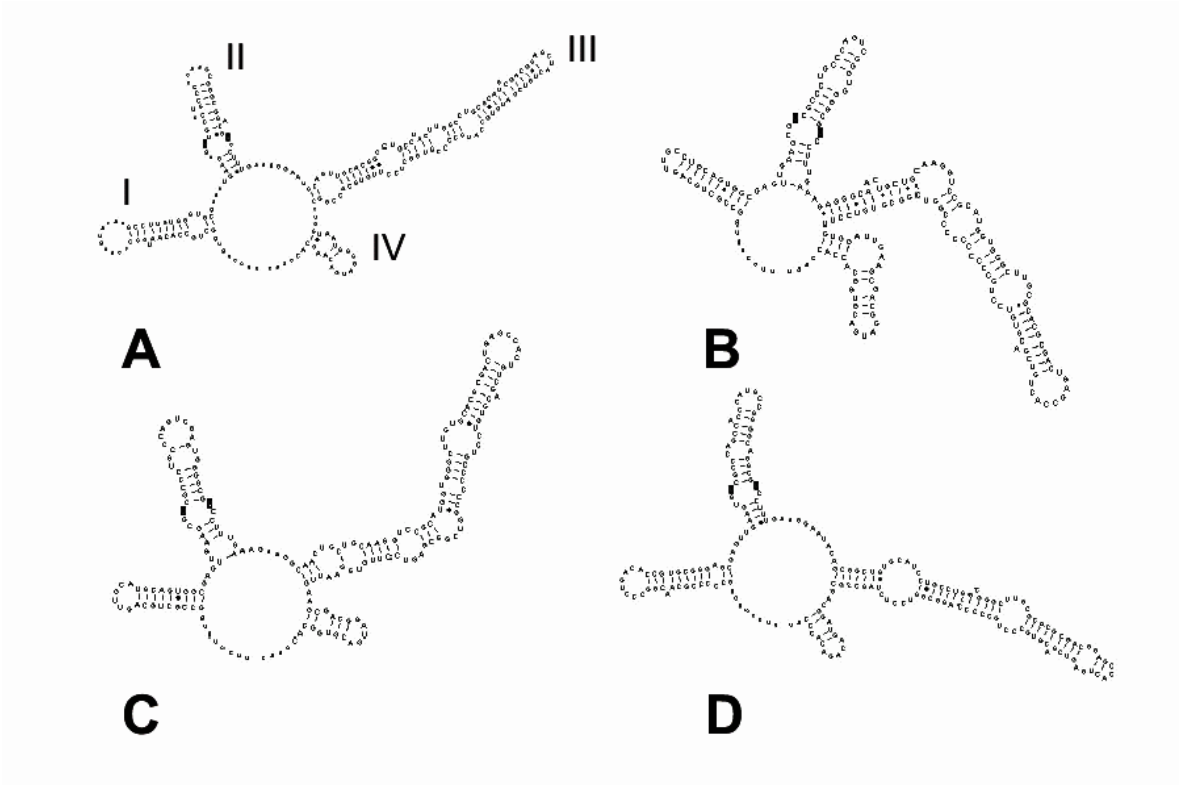

Fig. 3: Conserved secondary structure of the ITS-2 transcript in, e.g., *Verongula gigantea* (A), *Aplysina aerophoba* (B), *Aplysina lacunosa* (hard) (C), and *Aplysina lacunosa* (soft) (D). Universally conserved U-U mismatch in helix II is highlighted.

Because the 28S rRNA gene has also been reported to provide resolution for poriferan species (Alvarez et al. 2000), the primers RD3A and RD5B2 were used for amplification of an about 700 bp fragment of the 5' region of the 28S rRNA gene (McInerney et al. 1999). However phylogenetic analysis revealed an affiliation with the phylum Mollusca for 8 out of 10 sponge 28S rDNA consensus sequences analysed (data not shown). An affiliation with the Mollusca is also evident in sponge-derived sequences by McCormack et al. (2002) using the same primer set. The preference of primers for one phylogenetic group over another was previously described by Coleman for universal ITS primers (Coleman and Vacquier 2002). Because of such biases and previous difficulties in DNA amplification (Usher 2003) the primers RD3A and RD5B2 cannot be recommended for phylogenetic studies of sponges.

Table 2: Numerical and statistical values of the secondary structure (ITS-2) proposed in this study

Species	Sequence length (bases)	Loop length (unpaired bases)	GC content	Paired G-U in helices I-IV	Length of helices (in paired bases)				DG (37°C, kcal/mol)
					I	II	III	IV	
A. aerophoba	208	17	65%	10%	10	12	30	9	-91.0
A. cavernicola	210	23	63%	8%	10	13	27	9	-86.2
A. archeri	202	26	62%	7%	9	10	30	5	-81.9
A cauliformis (thin)	202	26	62%	6%	9	10	30	5	-83.9
A. cauliformis (thick)	202	26	61%	8%	9	10	30	4	-78.3
A. fistularis	202	26	61%	9%	9	10	30	5	-79.2
A. fistularis (CA)	202	26	60%	4%	7	10	32	5	-87.5
A. fulva	202	26	61%	7%	9	10	30	5	-81.9
A. insularis	201	26	62%	9%	9	10	30	5	-79.1
A. lacunosa (hard)	202	26	62%	6%	9	10	28	5	-80.7
A. lacunosa (soft)	205	29	68%	5%	11	13	30	4	-87.4
Verongula gigantea	210	31	60%	13%	11	12	29	4	-59.7

Neither 18S nor ITS-2 sequence data could completely resolve the phylogenetic relationships within *Aplysina* (Figs. 2, 3). The position of *A. lacunosa* (soft) basally outside the *Aplysina* cluster in both trees is surprising and suggests a possibly different phylogenetic placement.

Because extensive efforts were made to minimize sequencing errors this position is probably not a result of DNA amplification or sequencing errors. The position was furthermore verified for the ITS-2 phylogeny by a second ITS-2 sequence of another *A. lacunosa* (soft) individual collected a few days later from the same location. Moreover, sequencing of the mtDNA marker cytochrome oxidase subunit 1 (COI) of altogether seven *Aplysina* taxa confirmed both, the lack of resolution within *Aplysina* and the distant position of *A. lacunosa* (soft) within this group (data not shown). Additional evidence for a taxonomic reevaluation of *A. lacunosa* (soft) comes from subsequent morphological inspections of the sponge skeleton (S. Zea, data not shown). Whereas *A. lacunosa* (hard) possesses the typical *Aplysina* spongin skeleton, made up of a rather regular network of fibers with a thick, laminated bark and a thin, granular pith (see also Zea, 1987), *A. lacunosa* (soft) has a more open and irregular fiber skeleton of diverging and anastomosing fibers (hence, the softer consistency), with a rather thin bark in comparison to the pith. These features suggest an assignment possibly to the genus *Suberea* of the verongid family *Aplysinellidae* (see Bergquist and Cook 2002c).

Table 3: Number of compensatory base changes

	Aplysina aerophoba	*A. cavernicola*	*A. archeri*	*A. cauliformis* (thin)	*A. cauliformis* (thick)	*A. fistularis*	*A. fistularis* (CA)	*A.fulva*	*A. insularis*	*A. lacunosa* (hard)	*A. lacunosa* (soft)	*Verongula gigantea*
Aplysina aerophoba	-	1	9	10	9	9	9	9	9	8	12	26
A. cavernicola		-	9	9	9	9	9	9	8	10	15	25
A. archeri			-	0	0	0	0	0	0	0	18	25
A. cauliformis (thin)				-	0	0	0	0	0	0	18	25
A. cauliformis (thick)					-	0	0	0	0	0	18	26
A. fistularis						-	0	0	0	0	18	25
A. fistularis (CA)							-	0	0	0	20	26
A. fulva								-	0	0	18	25
A. insularis									-	0	18	25
A. lacunosa (hard)										-	18	24
A. lacunosa (soft)											-	21
Verongula gigantea												-

For the Caribbean *Aplysina* sponges and the Californian *A. fistularis* no further resolution could be obtained. The high genetic similarity raises the question of how far these sponges are phylogenetically separated. Recently, Coleman and co-workers correlated the ability of closely related taxa to interbreed with the numbers of Compensatory Base Changes (CBCs) that are observable in the secondary structure of the ITS-2 region (reviewed in Coleman 2000). CBCs occur in the intracellular paired regions of a primary RNA transcript when both nucleotides of a paired site mutate while the pairing itself is maintained (e.g. G-C mutate to A-U) (Gutell et al. 1994). Cross-fertilization experiments showed that taxa are sexually compatible when no CBCs have occurred. Accordingly, if a single CBC can be detected the taxa cannot interbreed and should therefore be considered as different species. While the Mediterranean sponges *A. aerophoba* and *A. cavernicola* show one CBC they are separated from the remaining *Aplysina* species by 8-10 CBCs (Table 3). Interestingly, the cluster from *A. archeri* to *A. fistularis* (CA) shows no CBCs at all. According to Coleman *A. aerophoba* and *A. cavernicola* are distinct, yet very closely related species whereas the Caribbean sponges plus the Californian *A. fistularis* represent sexually compatible taxa. This would imply that all remaining *Aplysina* sponges included here represent one single species. In consequence, ITS-2 sequence data then would resolve the relationships between the Mediterranean species *A. aerophoba* and *A. cavernicola* and a single Caribbean-Eastern Pacific *Aplysina* species. In fact it would be possible to test whether the Caribbean *Aplysina* can interbreed with cross-fertilization experiments but unfortunately only very few data about sexual reproduction of *Aplysina* sponges are available (Tsurumi and Reiswig 1997). However, as most of these species co-exist sympatrically in Caribbean reefs and have well defined and consistent morphologies, interbreeding does probably not occur. It is also conceivable that the occurrence of CBCs as an interbreeding barrier indicator may not be a universal phenomenon. Until more data become available it is preferable to maintain them as separate species owing to their clearly distinguishable morphotypes, their different distribution pattern along depth gradients and also due to their characteristic chemical profiles.

The clustering of *A. aerophoba* and *A. cavernicola* against all other *Aplysina* sponges in both trees (albeit with low bootstrap support) is in agreement with their geographical distribution. Assuming that the ITS-2 tree is more correct because it contains more *Aplysina* sequences, the following biogeographic scenario would be conceivable: a possibly Tethyan ancestor for all *Aplysina* resulted in a Mediterranean clade and in a Caribbean-Eastern Pacific clade with the latter being much more diverse than the former. If the progress of adaptive radiation resulting

in all extant Caribbean-Eastern Pacific *Aplysina* occurred only recently then this would explain the high degree of similarity between these genes. A similar phylogeographical history from the Mediterranean Sea to the Atlantic Ocean has recently been shown for *Crambe crambe* (Duran et al. 2004). For future studies it will be informative to include *A. aerophoba* from the Canary Islands to determine whether the Mediterranean and Atlantic populations are already distinguishable.

To our knowledge this is the first report of ITS-2 secondary structures for sponges. They were confirmed by the detection of several compensatory base changes in the helices while comparing *Aplysina* sponges and *Verongula gigantea* (Table 3). They are also in good accordance with the general folding pattern of ITS-2 found in a wide range of organisms showing a multibranch loop and four helices (e.g. Mai and Coleman 1997; Gottschling and Plötner 2004). For comparison an additional ITS-2 sequence of the non verongid sponge *Dysidea* sp. obtained from GenBank (Accession number: AF534700) was folded under the same conditions. The secondary structure was very similar to those presented in this study (data not shown). Therefore comparing the secondary structure of ITS-2 transcripts may help not only align sequences of close related sponge taxa but also of those representing higher taxonomic levels.

In conclusion, the resolution potential of 18S rDNA sequences was high enough to clearly resolve relationships between the genera *Aplysina*, *Aiolochroia* and *Verongula* but further resolution could not be obtained. The ITS-2 sequences resulted in a genetically indistinct Caribbean-Eastern Pacific *Aplysina* cluster and a Mediterranean cluster. Future studies such as nested clade analysis will provide insights into the phylogeography of this important and abundant marine sponge taxon.

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Chapter Three

Vertical transmission of microorganisms in Caribbean reef sponges

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I. Abstract

Caribbean reef sponges were surveyed for the presence of microorganisms in the mesohyl tissue of adult sponges and the respective reproductive material (embryos, larvae). A clear correlation was found in that high-microbial-abundance sponges always contained microorganisms in their reproductive stages. In contrast, low-microbial-abundance sponges did not contain microorganisms in their reproductive stages. Based on these data, *Ircinia felix* Duchassaing and Michelotti, 1864 was chosen as a model organism for the molecular documentation of vertical transmission of microorganisms. Denaturing gradient gel electrophoresis (DGGE) revealed similar banding patterns for *I. felix* adult and larvae whereas the juvenile banding pattern appeared as a mixture of sponge specific bands and bands representing seawater bacteria. Selected DGGE bands (n=21) were excised and sequenced. In total, 67% sponge specific sequences were obtained from each of the adult and larvae samples and 37.5% from the juveniles. In conclusion, high-microbial-abundance sponges transfer large microbial consortia vertically via their reproductive stages. For *I. felix* it could be proven that a large proportion of the transferred microorganisms belong to the sponge specific microbial community described previously.

II. Introduction

Sponges are filter-feeders that pump large volumes of seawater through their aquiferous system and take up food particles like organic particles and microorganisms by phagocytosis (Brusca and Brusca 1990). In addition to these food bacteria, sponges can permanently harbor large amounts of extracellular microorganisms in their mesohyl that make up 40-60% of the sponge biomass and exceed seawater concentrations by 2-4 orders of magnitude (Friedrich *et al.* 2001, Webster and Hill 2001). These sponge-associated microorganisms are morphologically diverse and often show unusual membrane structures like additional sheaths, slime layers or putative nuclear membranes (Fieseler *et al.* 2004, Friedrich *et al.* 1999, Fuerst *et al.* 1998, Vacelet and Donadey 1977, Wilkinson 1978).

16S rRNA gene based studies revealed phylogenetically complex, sponge-specific microbial consortia that are present in different sponges and that are remarkably different from seawater bacterioplankton, both in terms of concentration and diversity (Hentschel *et al.* 2003, 2006,

Hill 2004, Imhoff *et al.* 2003). In total, representatives of the eubacterial phyla Proteobacteria (Alpha-, Gamma- and Deltaproteobacteria), Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Nitrospira and Gemmatimonadetes (Friedrich *et al.* 1999, Hentschel *et al.* 2002, Schmitt *et al.* submitted, Taylor *et al.* 2005) and of the *Candidatus* phylum 'Poribacteria' (Fieseler *et al.* 2004) as well as of the archaeal phylum Crenarchaeota (Margot *et al.* 2002, Preston *et al.* 1996) were detected as specific members of the sponge microbiota. Moreover, it could be shown that the sponge-specific microbial consortia are stable over time and space (Hentschel *et al.* 2002). The microbial community profiles of *C. concentrica* were stable over large distances in temperate waters but differed between temperate and tropical seas (Taylor *et al*. 2005). The microbial consortia of verongid sponges were also stable after experimental perturbation (Friedrich *et al.* 2001, Thoms *et al.* 2003) however, microbial community variability was observed after copper exposure in *Rhopaloeides odorabile* Thompson, Murphy, Bergquist and Evans 1987 (Webster *et al*. 2001).

Vertical transmission has been proposed as a potential mechanism for the establishment and maintenance of this specific sponge-microbe-association. Microorganisms have already been detected by electron microscopy in oocytes of several oviparous sponges (e.g. *Aplysina* Nardo, 1834 (Gallissian and Vacelet 1976), *Stelletta grubii* Schmidt, 1862 (Sciscioli *et al.* 1991), *Geodia cydonium* Jameson, 1811 (Sciscioli *et al.* 1994), *Chondrilla* Schmidt, 1862 sponges (Maldonado *et al.* 2005)) and in embryos and larvae of several viviparous sponges (e. g. *Spongia* Linneaus, 1759 and *Hippospongia* Schulze, 1879 (Kaye 1991, Kaye and Reiswig 1991), *Chondrosia reniformis* Nardo, 1847 (Levi and Levi 1976)). Usher *et al.* (2001) and Ereskovsky *et al.* (2005) reported in detail on the incorporation and transmission of Cyanobacteria in *Chondrilla australiensis* Carter, 1873 and of a spiral bacterium in *Halisarca dujardini* Johnston, 1842, respectively. Furthermore, Enticknap *et al.* (2006) succeeded in cultivating several alphaproteobacterial strains from larvae of the sponge *Mycale laxissima* Duchassaing and Michelotti, 1864. These bacteria were closely related to each other and also to the strain NW001 isolated from the sponge *R. odorabile* (Webster and Hill 2001).

To obtain a better understanding of the establishment and maintenance of this unique association between sponges and microbial consortia we performed a general electronmicroscopical survey for the presence of microorganisms in adult sponges as well as in the respective reproductive stages. In total, ten Caribbean species representing five orders

and two different modes of reproduction (ovipary, vivipary) were included. Based on these results, *Ircinia felix* Duchassaing and Michelotti, 1864 was chosen as a model system for the detailed molecular study of vertical transmission. *I. felix* is a viviparous species with internal fertilization. Embryos are brooded in the sponge mesohyl and free swimming larvae spend a few hours in the water column before they settle on a suitable substrate and metamorphose into juveniles. We performed settlement experiments on reefs offshore Florida and applied DGGE analysis to phylogenetically characterize and compare the associated microbiota of three different developmental stages (adult, larvae, and juveniles).

III. Materials and Methods

Adult and reproductive material of ten sponge species was collected by SCUBA diving offshore off Key Largo, Florida in June 2002 and June and August 2004 using the NOAA´s National Undersea Research Center (NURC) facilities and vessels. For TEM, small pieces of each sample were fixed in 2.5% glutaraldehyde/H_2O, postfixed in 2% osmium tetroxide and incubated in 0.5% uranyl acetate. After dehydration in an ethanol series, samples were incubated in 1x propylene oxide and finally embedded in Epon 812. The samples were sectioned with an ultramicrotom (OM U3, C. Reichert, Austria) and pieces of three different individuals of each sponge developmental stage (except for *A. coralliphagum* embryos where only one sample was available) were examined by transmission electron microscopy (Zeiss EM 10, Zeiss, Germany). Additionally, settlement experiments were performed with the sponge *I. felix*. Larvae were caught using the methodology of Lindquist *et al.* (1997), transferred into sealed plastic containers (~ 75ml volume) and returned to the reef where they were cable tied to racks at 9m depth. After settlement, pieces of the Nylon with settled juvenile sponges as well as pieces of Nylon without sponge tissue (control) were collected. DGGE was performed with all samples as described in Schmitt *et al.* (submitted). Briefly, DNA was extracted using the Fast DNA Spin Kit for Soil (Q-Biogene, Heidelberg, Germany) or by heating (100°C) of the samples. Following PCR amplification with universal, eubacterial 16S rDNA-targeted primers 341f with GC clamp and 907r (Muyzer *et al.* 1998), the resulting PCR products were run on a 10% (w/v) polyacrylamide gel that was stained afterwards with SYBR Gold (Molecular Probes) and analyzed using Quantity One (Bio-Rad, München, Germany). Selected bands were excised, cloned and sequenced on an ABI automated sequencer.

Table 1: Detection of microorganisms in adult sponges and reproductive stages using electron microscopy.

Species	Order	Detection of microorganisms by TEM	
		adult	reproductive stages
High microbial abundance sponges			
Agelas wiedenmayeri Alcolado, 1984	Agelasida	+	+
Aka coralliphagum Ruetzler, 1971	Haplosclerida	+	+
Ectyoplasia ferox Duchassaing & Michelotti, 1864	Poecilosclerida	+	+
Ircinia felix Duchassaing & Michelotti, 1864	Dictyoceratida	+	+
Smenospongia aurea Hyatt, 1875	Verongida	+	+
Low microbial abundance sponges			
Callyspongia vaginalis Lamarck, 1814	Haplosclerida	–	–
Mycale laxissima Duchassaing & Michelotti, 1864	Poecilosclerida	–	–
Niphates digitalis Lamarck, 1814	Haplosclerida	–	–
Tedania ignis Duchassaing & Michelotti, 1864	Poecilosclerida	–	–
Ulosa ruetzleri Wiedenmayer, 1977	Poecilosclerida	–	–

IV. Results

Transmission Electron Microscopy

High-microbial-abundance sponges

In adult samples of *Agelas wiedenmayeri* Alcolado, 1984, *Aka coralliphagum* Ruetzler, 1971, *Ectyoplasia ferox* Duchassaing & Michelotti, 1864, *I. felix* and *Smenospongia aurea* Hyatt, 1875 large numbers of extracellular microorganisms were scattered throughout the sponge mesohyl (Table 1, Fig.1A, C, E, G, J). These sponges are therefore regarded as high-microbial-abundance species. The microorganisms showed a high variety of morphotypes, such as rods, cocci and other, irregular forms. Many microorganisms possessed additional membrane structures. Cyanobacteria could be identified by their typical thylacoid membranes and were particularly dominant in the *I. felix* mesohyl (Fig.1G). Some loosely scattered sponge cells were also present in the mesohyl. Most of these cells were amoeboid-like and contained large nuclei and often vesicles and phagosomes showing their phagocytotic activity. A layer of pinacocytes and/or choanocytes always separated the mesohyl of these sponges from seawater.

A. coralliphagum, I. felix and *S. aurea* have a viviparous mode of reproduction and release free swimming parenchymella-type larvae into the water column. Many microorganisms were

predominantly located in the central region of the larvae (Fig.1D, H, K). These morphologically diverse microorganisms were extracellular and similar in shape to the microorganisms present in the respective adult tissues. Few amoeboid-like sponge cells, that contain large amounts of lipids, were also present in the center of the larvae. *A. wiedenmayeri* and *E. ferox* have an oviparous mode of reproduction. They release oocytes or zygotes, which are embedded in a gelatinous sheath. These early reproductive stages were densely filled with lipids and electron-dense vesicles (Fig.1B, F). Microorganisms were predominantly found in the outer regions of the reproductive stages of *A. wiedenmayeri* and *E. ferox*. (Fig.1B, F).

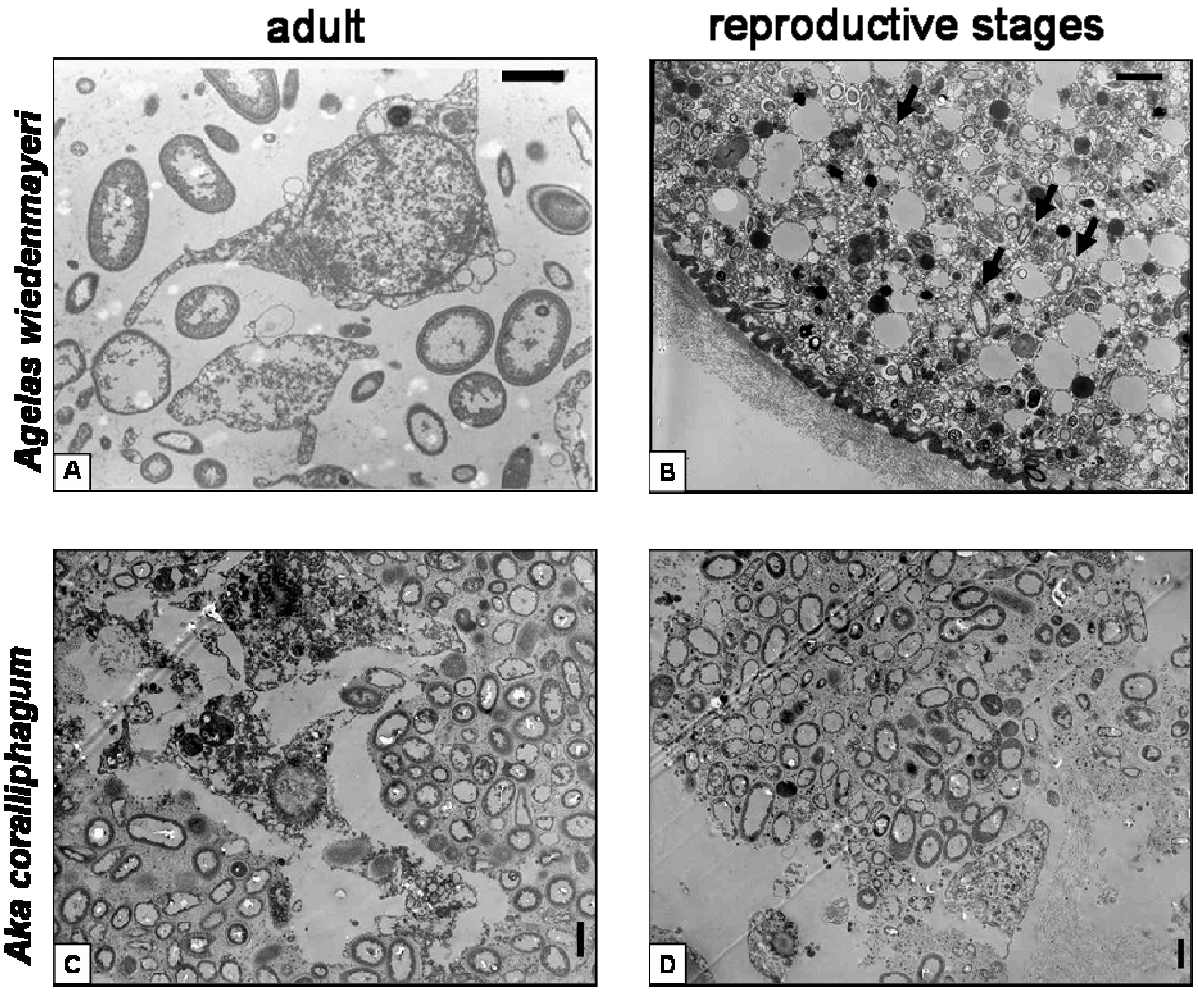

Fig. 1: Transmission electron microscopy of the high-microbial-abundance sponges *A. wiedenmayeri* adult (A) and embryo (B), *A. coralliphagum* adult (C) and embryo (D), *E. ferox* adult (E) and embryo (F), *I. felix* adult (G) and larvae (H) and *S. aurea* adult (J) and larvae (K). Arrows indicate microorganisms. Cy: Cyanobacteria. Scale bar: 1µm (A, C, D, G, H, J, K), 2µm (B, E, F).

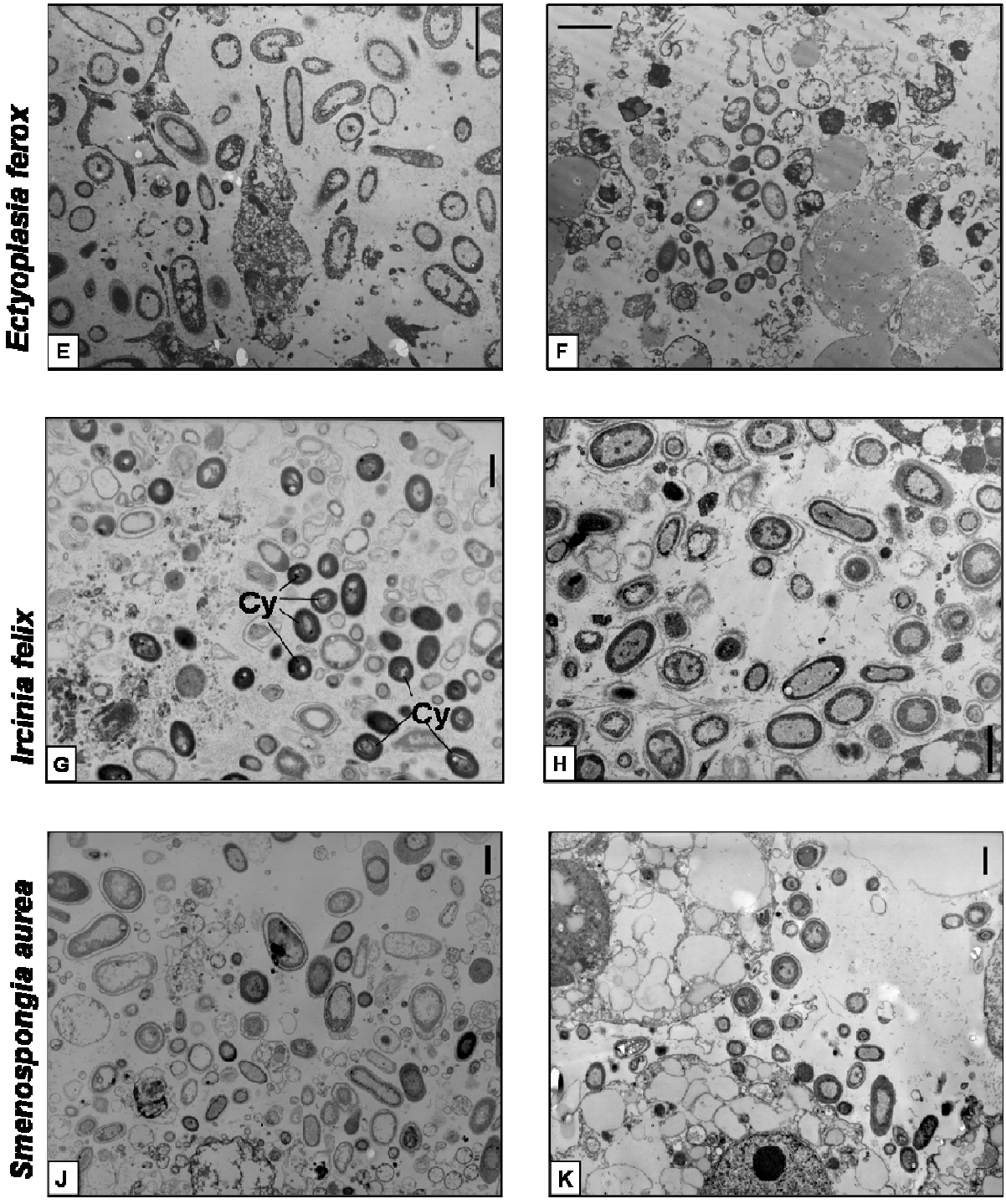

Fig. 1: continued.

Low-microbial-abundance sponges

EM inspection of *Callyspongia vaginalis* Lamarck, 1814, *M. laxissima, Niphates digitalis* Lamarck, 1814, *Tedania ignis* Duchassaing & Michelotti, 1864 and *Ulosa ruetzleri* Wiedenmayer, 1977 adult samples revealed the complete absence of microorganisms in the mesohyl matrix (Table 1, Fig.2A, C, E, G, J) and are therefore classified as low-microbial-abundance sponges. The mesohyl contained few sponge cells that were embedded in a voluminous extracellular matrix. All investigated species are viviparous. Their larvae contained high numbers and sometimes very large vesicles and also many lipids, but microorganisms could not be detected in these larvae (Fig.2B, D, F, H, K).

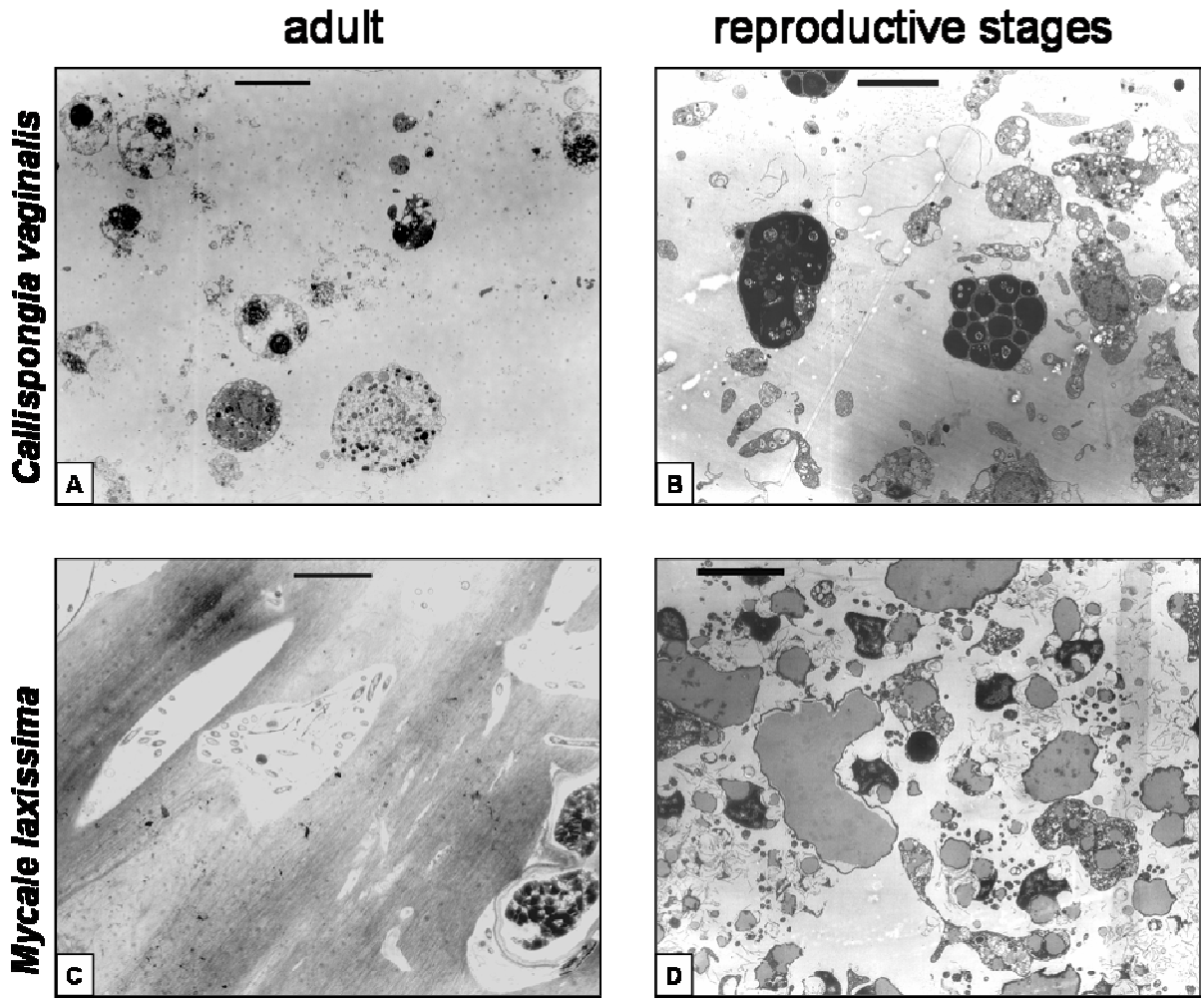

Fig. 2: Transmission electron microscopy of the low-microbial-abundance sponges *C. vaginalis* adult (A) and larvae (B), *M. laxissima* adult (C) and larvae (D), *N. digitalis* adult (E) and larvae (F), *T. ignis* adult (G) and larvae (H) and *U. ruetzleri* adult (J) and larvae (K). Scale bar: 3µm (K), 4µm (F), 5µm (A, B, C, D, E, G, H), 8µm (J).

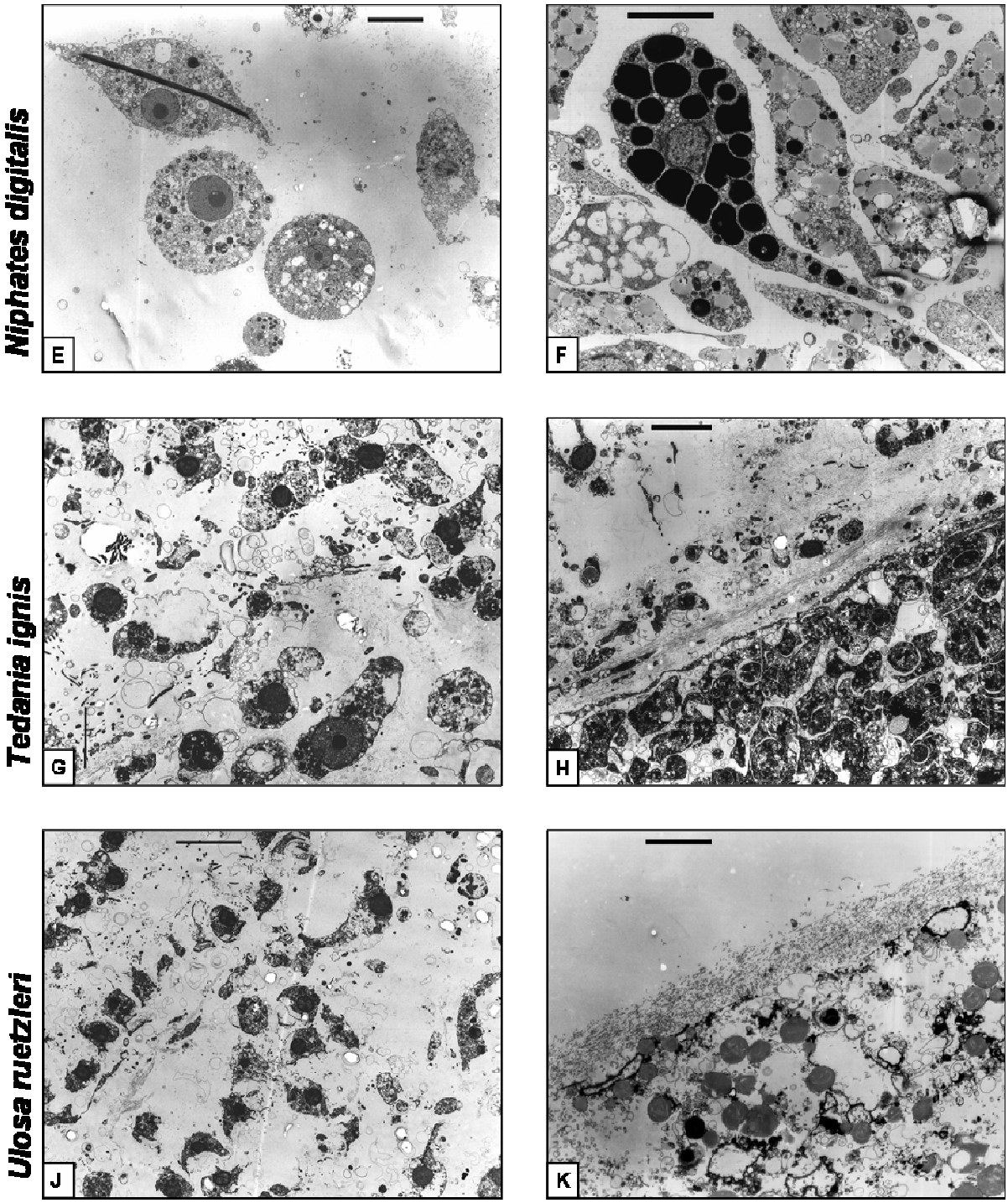

Fig. 2: continued.

Denaturing Gradient Gel Electrophoresis (DGGE)

Fig. 3A represents the bacterial profiles of *I. felix* adult, larva and juvenile as well as the control (piece of Nylon without sponge tissue). Two independent PCR reactions of each sponge sample were run on the gel to test for PCR biases. The DGGE banding patterns of the two adult, larvae, and juvenile samples differed in only one, four, and two band positions, respectively, indicating that a PCR bias is negligible. The number of bands in *I. felix* adult was higher than in the larvae (adult n =20.5; larva n=16), but the DGGE banding patterns were highly similar. Overall, adult and larvae samples shared more than 70% of all bands (Fig. 3B). The juvenile sample differed from the adult and larva samples in that it had generally less bands (n=13) and shared only 53% of all bands with adult and larvae. The cluster analysis placed the juvenile sample to the control (number of bands: n=17) together, albeit with only 54% similarity (Fig. 3B).

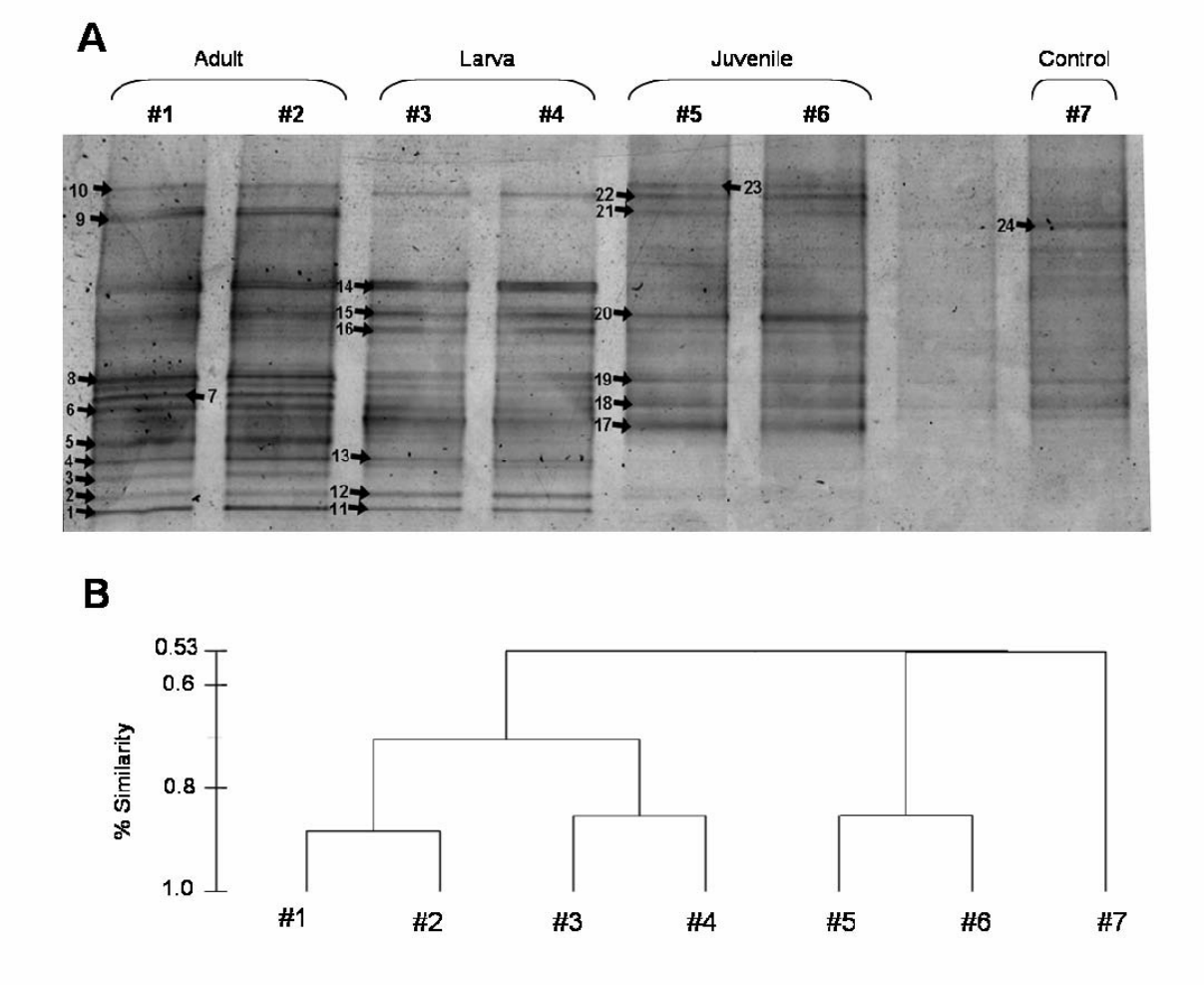

Fig. 3: (A): 16S rDNA-DGGE gel of *I. felix* adult, larvae and juvenile samples as well as a Nylon-control sample. Two independent PCR reactions were run for each sample. Arrows mark excised bands. (B): Cluster analysis of the DGGE gel showing percentage similarity of banding patterns.

16S rDNA sequence analysis

In total, 21 16S rRNA gene sequences were obtained from the *I. felix* DGGE gel: nine from adult, three from larva, eight from juvenile, and one additional sequence from the control (Fig. 3A, Table 2). The overall diversity was high with representatives of four different bacterial phyla (Gemmatimonadetes, Acidobacteria, Proteobacteria (Alpha-, Gamma-, Deltaproteobacteria) and Chloroflexi). In the adult sample, seven sequences were most similar to sequences derived from the sponges *Aplysina aerophoba* Schmidt, 1862, *Aplysina cavernicola* Vacelet, 1959, *Theonella swinhoei* Gray, 1868 and *Cymbastela concentrica* Lendenfeld, 1887. Clone B8-1 was related to a gastropod gill endosymbiont and clone B10-1 was related to an uncultured *Rhodovulum* CtaxPhil-1. In the larva, two sequences were most similar to a 16S rRNA gene sequence from *A. cavernicola*, whereas clone B13-1 was related to a sea water clone. The juvenile sample contained three 16S rRNA gene sequences most similar to sequences derived from the sponge *A. aerophoba*, two sequences most similar to *Alcanivorax* sp., and three sequences most similar to a cold seep clone, a coral associated bacterium, and an uncultured *Rhodovulum* CtaxPhil-1, respectively. The 16S rRNA gene sequence obtained from the control was related to a marine *Pseudoalteromonas* sp. sequence (Table 2).

V. Discussion

The EM survey for the presence of microorganisms in the sponge mesohyl yielded two different groups of sponges. One group contained large numbers of morphologically diverse microorganisms whereas the mesohyl of the second group was completely devoid of microorganisms. These data expand early observations on patterns of microbial abundances in sponges by Vacelet (1975) and Vacelet and Wilkinson (1977). Whenever microorganisms were present in the adult sponge, microorganisms were also contained in the respective reproductive stages (Table 1, Fig. 1). Whenever microorganisms were absent in the adult sample, microorganisms were also missing in the reproductive stages (Table 1, Fig. 2). This correlation strongly suggests that high-microbial-abundance sponges transfer microorganisms vertically through their reproductive stages. Furthermore, the morphological diversity of the microorganisms in reproductive stages indicates that complex microbial consortia rather than single lineages are transferred. Apparently, vertical transmission is common and widespread among high-microbial-abundance sponges.

Table 2: 16S rDNA sequence analysis of bands excised from the DGGE gel of *I. felix*.

	Clone	Closest sequence match in GenBank	Similarity (%)	Length (bp)	Taxonomic affiliation
adult	B1-1	sponge clone PAUC43f (AF186415)	99	585/589	Gemmatimonadetes
	B2-1	sponge DGGE band 6 (AY180081)	97	487/499	Acidobacteria
	B3-1	sponge clone TK19 (AJ347028)	96	567/589	Gemmatimonadetes
	B5-1	sponge clone TK13 (AJ347034)	98	577/588	Deltaproteobacteria
	B6-1	sponge clone TK13 (AJ347034)	94	557/588	Deltaproteobacteria
	B7-2	sponge clone Cc007 (AY942754)	93	541/576	Gammaproteobacteria
	B8-1	gastropod endosymbiont (AB214932)	91	537/587	Gammaproteobacteria
	B9-1	sponge clone TK16 (AJ347035)	97	548/564	Chloroflexi
	B10-1	uncult. *Rhodovulum* CtaxPhil-1 (AF259621)	92	518/560	Alphaproteobacteria
larvae	B11-1	sponge DGGE Band 6 (AY180081)	97	487/499	Acidobacteria
	B12-1	sponge DGGE Band 6 (AY180081)	97	486/499	Acidobacteria
	B13-1	sea water clone (AY592226)	96	569/589	Acidobacteria
juvenile	B17-1	sponge clone TK34 (AJ347030)	98	548/559	Alphaproteobacteria
	B18-1	cold seep clone (AB015247)	94	530/560	Alphaproteobacteria
	B18-3	sponge clone TK97 (AJ347054)	96	529/547	Alphaproteobacteria
	B18-4	sponge clone TK34 (AJ347030)	98	551/559	Alphaproteobacteria
	B19-2	marine *Alcanivorax* sp. (AY726812)	89	502/561	Alphaproteobacteria
	B20-1	coral associated bacterium (DQ200552)	91	551/593	Gammaproteobacteria
	B22-1	uncult. *Rhodovulum* CtaxPhil-1 (AF259621)	91	515/561	Alphaproteobacteria
	B23-1	*Alcanivorax* sp. Mho1 (AB053124)	99	586/587	Gammaproteobacteria
control	B24-1	*Pseudoalteromonas* sp. (AM111085)	96	472/489	Gammaproteobacteria

The mode of reproduction seems not to be a determining factor for vertical transmission as both oviparous (*A. wiedenmayeri, E. ferox*) and viviparous (*A. coralliphagum*, *I. felix, S. aurea*) species belong to the high-microbial-abundance group (Table 1). This is consistent with previous electron microscopy studies that also documented the presence of microorganisms in oocytes of oviparous species (Gallissian and Vacelet 1976, Usher *et al.* 2001) and in oocytes and embryos of viviparous species (Ereskovsky *et al.* 2005, Kaye 1991). This further supports the general character of the microbial transfer through larvae in sponges.

The high-microbial-abundance sponge *I. felix* was chosen for further molecular investigations. DGGE was performed to compare the bacterial profiles of different sponge developmental stages. *I. felix* larvae contained a highly similar microbial community as the adult sponge indicated by a highly similar banding pattern (Fig. 2). Apparently, a large proportion of the adult microbial community is vertically transmitted in this species. The juvenile banding pattern is more variable and resembles partly the other sponge samples and partly the control banding pattern. This might be an artefact from the extraction protocol as the juveniles were still attached to the nylon substrate at the time of DNA extraction. Accordingly, the DGGE banding pattern is a mixture of sponge-specific bands and bands of colonizers from seawater. Additionally, five bands are exclusively found in the juvenile sample. They might represent seawater bacteria which have been taken up as food source. The presence of choanocyte chambers in the electron micrographs of juveniles shows that the aquiferous system has already been established and the juveniles are therefore able of pumping water (data not shown).

We excised and sequenced representative bands from the DGGE gels and found, that the phylogenetic diversity in *I. felix* resembles that of other high-microbial-abundance sponges (for recent reviews see Hentschel *et al.* 2003 and 2006, Hill 2004, Imhoff and Stöhr 2003). In total, 60% of all sequences from *I. felix* are most similar to a sequence derived from another sponge. This proportion of the whole community is regarded to be sponge-specific. Sponge-specific sequences dominated in adult and larval stages whereas in the juvenile a higher percentage of environmental sequences were found. Therefore, *I. felix* is a typical representative of the high-microbial-abundance sponge group with a large, complex and highly sponge-specific microbial community. This microbial community is transferred vertically via the larvae to the next sponge generation.

In summary, the TEM survey revealed that the Caribbean sponges *A. wiedenmayeri*, *A. coralliphagum*, *E. ferox*, *I. felix* and *S. aurea* are associated with large amounts of microorganisms and that these microorganisms are most likely transferred vertically via the sponge reproductive stages. Other sponges that coexist in the same habitat, (*C. vaginalis*, *M. laxissima*, *N. digitalis*, *T. ignis* and *U. ruetzleri*) neither contain microorganisms neither in the adult mesohyl nor in the corresponding larvae. DGGE sequence analysis of adult, larvae and juvenile samples of *I. felix* revealed that representatives of the previously described sponge specific microbial consortium (Hentschel et al. 2002) are present in *I. felix* and are transferred via the larvae. Therefore, vertical transmission is clearly important to establish and maintain the phylogenetically complex yet highly sponge-specific microbial community in *I. felix* and probably also in many other marine high-microbial-abundance sponges.

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Chapter Four

Vertical transmission of a phylogenetically complex microbial consortium in the viviparous sponge *Ircinia felix*

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I. Abstract

Many marine demosponges contain large amounts of phylogenetically complex, yet highly sponge-specific microbial consortia within the mesohyl matrix but little is known how these microorganisms are acquired by their hosts. Settlement experiments were performed with the viviparous Caribbean demosponge *Ircinia felix* to investigate the role of larvae for vertical transmission of the sponge-associated microbial community. Inspections by electron microscopy revealed large amounts of morphologically diverse microorganisms in the center of *I. felix* larvae while the outer rim appeared to be devoid of microorganisms. In juveniles, microorganisms were found between densely packed sponge cells. Denaturing gradient gel electrophoresis (DGGE) was performed to compare the bacterial community profiles of adults, larvae and juvenile sponges. Adults and larvae were highly similar in DGGE band numbers and banding patterns. Larvae released by the same adult individual contained highly similar DGGE banding patterns whereas larvae released by different adult individuals showed slightly different DGGE banding patterns. Over 200 bands were excised, sequenced and phylogenetically analysed. The bacterial diversity of adult *I. felix* and its larvae was comparably high while juveniles showed reduced diversity. In total, thirteen vertically transmitted sequence clusters, hereafter termed 'IF clusters', were found that contained sequences from both, the adult sponge and offspring (larvae and/ or juveniles). The IF clusters belonged to at least four different eubacterial phyla and one possibly novel eubacterial lineage. In summary, it could be shown that in *I. felix*, vertical transmission of microorganisms through the larvae is an important mechanism for the establishment of the sponge-microbe association.

II. Introduction

Sponges (*Porifera*) are evolutionarily ancient Metazoa that first appeared in Precambrian times nearly 600 million years ago (Li et al. 1998). Today, an estimated 13,000 species, classified in three classes (*Demospongiae*, *Calcarea*, *Hexactinellida*) populate virtually all aquatic habitats from shallow tropical reefs to the polar seas and the deep ocean and even freshwater lakes and rivers (Hooper and van Soest 2002). A great diversity of sponges occurs on coral reefs, where they exhibit a wide range of shapes and colors. Sponges have a primitive morphology as they lack true organs or tissues. Instead, sponges possess totipotent, mobile

cells freely scattered in an extracellular matrix called the mesohyl, which is covered by a single cell layer, the pinacoderm. Inhalant and exhalant canals in the mesohyl build an aquiferous system through which water is pumped actively by flagellated choanocytes (Brusca and Brusca 1990). As filter-feeders, sponges efficiently take up nutrients like organic particles and microorganisms from the seawater, leaving the expelled water essentially sterile (Pile 1997, Reiswig 1974, Wehrl et al. in press). Reproduction occurs either oviparously or viviparously. In oviparous sponges, gametes are released into the water during large, synchronized spawning events with fertilization occurring outside the sponge body. Eggs of viviparous sponges are fertilized internally, and the brooding of embryos occurs within the mesohyl, often in specialized brooding chambers. After their release, larvae of viviparous sponges actively swim for a relatively short period of time (Lindquist et al. 1997) or creep over the substrate in search of suitable settlement sites. Upon settlement, they undergo a rapid metamorphosis to an early juvenile stage. Additionally, many sponges also reproduce asexually via budding or the formation of gemmulae.

The association of many demosponges with large microbial consortia is well recognized (Hentschel et al. 2003, 2006, Hill 2004, Imhoff and Stöhr 2003). This complex microbial community differs significantly from seawater and sediments in both, concentration and diversity (Friedrich et al. 1999, Hentschel et al. 2002, Webster et al. 2001). 40- 60% of sponge biomass can consist of microorganisms which are mostly located extracellularly in the mesohyl. This equals 10^8- 10^{10} bacteria g^{-1} tissue and exceeds seawater concentrations by two to four orders of magnitude (Friedrich et al. 2001, Webster and Hill 2001). Morphological diversity of sponge associated microorganisms was shown by electron microscopy. Several different morphotypes with unusual membrane features were described in various sponge species (Friedrich et al. 1999, Fuerst et al. 1998, Vacelet 1975, Vacelet and Donadey 1977, Willenz and Hartman 1989). More recently, classical cultivation as well as molecular studies based on 16S rRNA gene sequence information revealed a high phylogenetic complexity of sponge-associated microbial consortia. Members of eight phyla within the domain *Bacteria* [*Proteobacteria* (*Alpha-, Gamma-, Deltaproteobacteria), Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Gemmatimonadetes* and *Nitrospira*] and one archaeal phylum (*Crenarchaeota*) have been detected (Hentschel et al. 2002, Olson and McCarthy 2005, Preston et al. 1996, Taylor et al. 2005, Thoms et al. 2003, Webster et al. 2001). Further, a new candidate phylum, termed *Poribacteria*, has been detected in several high microbial abundance sponges (Fieseler et al. 2004, 2006). This phylogenetically complex

microbial consortium is highly sponge-specific in that the corresponding phylogenetic lineages have been found in taxonomically and geographically different marine demosponges but have not been detected in seawater, sediments, in other marine invertebrates or a freshwater sponge (Gernert et al. 2005).

Presently, little is known about how the unique and apparently stable sponge-microbial associations are established and maintained over time. Vertical transmission as a mechanism for bacterial passage between sponge generations was already proposed by Levi and Porte in 1962. Electron microscopy studies revealed the presence of microorganisms in oocytes of oviparous sponges and embryos/larvae of viviparous sponges (Ereskovsky et al. 2005 and references cited therein, Kaye 1991, Kaye and Reiswig 1991, Levi and Levi 1976, Ruetzler et al. 2003, Sciscioli et al. 1994). Vertical transmission of *Cyanobacteria* in developing eggs and sperm of the sponge *Chondrilla australiensis* (Usher et al. 2001) and vertical transmission of a spiral bacterium through all stages of embryonic development in the sponge *Halisarca dujardini* (Ereskovsky et al. 2005) was reported. Similarly, Maldonado and coworkers (Maldonado et al. 2005) used electron microscopy to document the transmission of yeast in *Chondrilla* species. Enticknap et al. (Enticknap et al. 2006) were the first to phylogenetically identify alphaproteobacteria associated with larvae of the sponge *Mycale laxissima* and to confirm their presence by fluorescence *in situ* hybridisation. The isolates JE061-JE065 were most closely related to strain MBIC3368 that had previously been isolated from at least eight other demosponges (Scheuermayer et al. 2006). Sharp et al. (2007) reported on vertical transmission of complex bacterial and archaeal consortia in the tropical sponge, *Corticium* sp.. Using fluorescently labelled 16S rRNA probes, it was possible to localize specific microbial lineages in the adult and in early and late stages of embryonic development.

For this study, the ball-shaped sponge *Ircinia felix* Duchassaing and Michelotti 1864 (order *Dictyoceratida*) was chosen because it is a common and accessible species on shallow Caribbean coral reefs, grass beds and mangroves. It is easily identified by its grey to light brown color and hexagonally oriented, low white knobs that are interconnected by white lines. *Ircinia* species produce an array of low-molecular-weight volatile compounds that gives them a strong, pungent smell (Pawlik et al. 2002). *Ircinia* sponges are viviparous and produce relatively large parenchymella-type larvae. In the Florida Keys they are released in the early morning hours (0700-1000h) in synchronized spawning events 1-2 days following the full moon typically in May and June (N. Lindquist, personal observation). Larvae swim briefly

after spawning and settle within minutes to several hours and then metamorphose to a flattened early juvenile stage within a day. The aim of this study was to identify, localize and phylogenetically characterize the bacterial community in adult, larvae and early stage juveniles of *I. felix*. The different developmental stages were analyzed by a combination of molecular (denaturing gradient gel electrophoresis) and visual (electron microscopy) techniques.

III. Materials and Methods

Larvae settlement experiments

Experiments were performed on a shallow tropical patch reef (25°01.610'N / 80°23.671'W) offshore Key Largo, Florida in June and August 2004 using the NOAA´s National Undersea Research Center (NURC) facilities and vessels. Sponge larvae were collected *in situ* from four individual sponges (#2, 3, 4, 5) using the methodology of Lindquist et al. (1997) and transferred into 3L holding trays containing natural seawater. Additionally, tissue samples were taken from the corresponding adult specimens from whom larvae had been collected. Individual larvae were captured from the holding trays with glass Pasteur pipettes and transferred either into 150µl sterile filtered seawater for DNA extraction and stored at -80°C or were fixed in 2.5% glutaraldehyde/H_2O_{dd} for transmission electron microscopy and stored at 4°C. Larvae not used for microscopy or DNA extraction were settled by placing them in sealed plastic containers (~75 ml vol) with NITEX (100 µm) "windows" to allow for the flow of ambient seawater into the containers. *I. felix* larvae settled on the nylon mesh (i.e., pantyhose) that held the larval collection containers on a rack at a depth of 9 m. During this set up process, larval and juvenile contact with air was avoided. Juveniles were recovered 1-3 day post-settlement, along with control pieces of mesh, which did not contain settled sponges. These samples were preserved as described above for DNA and microscopic analysis. Fluorescence *in situ* hybridisation (FISH) experiments could not be reliably performed because of the small larval size and limited juvenile biomass.

Transmission Electron Microscopy (TEM)

The adult sponge samples that had been preserved in 2.5% glutaraldehyde/H_2O_{dd} were cut into small pieces of about $1mm^3$. All samples (adult, larvae, juveniles) were then washed five times in cacodylate buffer (50 mM, pH 7.2), fixed in 2% osmium tetroxide for 90 min,

washed again five times in H_2O_{dd} and incubated overnight in 0.5% uranyl acetate. After dehydration in an ethanol series (30, 50, 70, 90, 96, 3x100% for 30 min, respectively), samples were incubated 3x30 min in 1x propylene oxide, overnight in 1:1 (v/v) propylene oxide/Epon 812 (Serva), 2x2 h in Epon 812 and finally embedded in Epon 812 for 48 hours at 60°C. Samples were then sectioned with an ultramicrotom (OM U3 C. Reichert, Austria) and examined by transmission electron microscopy (Zeiss EM 10, Zeiss, Germany).

Denaturing Gradient Gel Electrophoresis (DGGE)

Frozen pieces of adult sponge tissue were ground in liquid nitrogen with a mortar and pestle and DNA was extracted using the Fast DNA Spin kit for soil (Q-Biogene, Heidelberg, Germany) in accordance with the manufacturer's instructions. Larvae and juvenile DNA was extracted from 3, 5 or 7 individual larvae or juveniles, which had been pooled immediately after collection, by heating in 150µl H_2O_{dd} in a water bath for 10 min at 100°C. The solution was used as template for PCR. The juvenile DNA was extracted together with a small piece of nylon on which the sponge had grown because it was impossible to remove the tiny sponge from its substratum without losing too much biomass. The universal primers 341f with GC-clamp and 907r (Muyzer et al. 1998) were used for PCR amplification of bacterial 16S rDNA. Cycling conditions on a Mastercycler Gradient (Eppendorf, Hamburg, Germany) were as follows: initial denaturing step at 95°C for 5 min, 30 cycles of denaturing at 95°C for 1 min, primer annealing at 54°C for 1 min and elongation at 72°C for 45 sec, followed by a final extension step at 72°C for 10 min. DGGE was performed with a Bio-Rad DCode Universal Mutation Detection System (Bio-Rad, München, Germany) on a 10% (w/v) polyacrylamide gel in 1x TAE and using a 0–90% denaturing gradient; 100% denaturant corresponded to 7 M urea and 40% (v/v) formamide. Electrophoresis was performed for 6 h at 150 V and 60°C. Gels were stained for 30 min in SYBR Gold (Molecular Probes) and scanned on a Typhoon 8600 scanner (Amersham Biosciences). DGGE banding pattern similarities were determined by cluster analysis using Quantity One (Bio-Rad, München, Germany). Selected bands were excised with an EtOH sterilized scalpel and incubated in 25µl H_2O_{dd} overnight at 4°C. 4 µl of eluted DNA was subsequently used for reamplification with primers 341f and 907r under PCR conditions described above. PCR products were ligated into the pGEM-T-easy vector (Promega) and transformed by electroporation into competent *E. coli* XL 1-Blue cells. Plasmid DNA of up to four different clones per excised band was isolated by standard miniprep procedures and the correct insert size was verified by using agarose gel electrophoresis following restriction digestion (Sambrook and Russell 2001). Sequencing was

performed on an ABI 377XL automated sequencer (Applied Biosystems). Sequences were edited with the ContigExpress Tool in Vector NTI suite 6.0 (InforMax, Inc).

Phylogenetic sequence analysis

Sequences were checked for chimeras with the program Pintail (Ashelford et al. 2005) and for other amplification and sequencing artifacts. Following removal of chimeras from the dataset, percentage similarities (p-distances) between sequences from the same source (adult, larvae, juvenile) were determined with the editor Align (Multicolor sequence Alignment Editor. Distributed by the author. Available from: http://wwwuser.gwdg.de/~dheppner/) and those with identities above 99% were grouped together in operational taxonomic units (OTUs). Only one randomly chosen sequence per OTU was used for further analysis. As a first approximation, the phylogenetic affiliation was determined for each OTU by comparison against sequences available in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Sequences obtained in this study together with reference sequences (all nearest BLAST matches and, moreover, representatives of the respective phylum) were aligned automatically with ClustalX (Thompson et al. 1997) and the alignment was subsequently corrected manually in Align. Phylogenetic trees were constructed with the ARB software package (Ludwig et al. 2004). Initially, neighbour-joining (Jukes-Cantor correction) and maximum parsimony trees were calculated with nearly full length sequences (>1250bp) and 100 pseudoreplicates. Subsequently, partial sequences were added to the trees without changing the topology by the use of the parsimony-interactive method in ARB. Finally, 50% majority rule consensus trees were constructed.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained in this study were deposited in EMBL/GenBank/DDBJ under accession numbers DQ661746-DQ661857.

IV. Results

Transmission electron microscopy

Electron microscopy of adult *I. felix* #4 and #5 revealed large and complex microbial communities (Fig. 1). Microorganisms are embedded extracellularly and are scattered equally in the collagen matrix of the mesohyl. Bacterial sizes are between 1 and 2.5μm in length and

0.5 to 1µm in diameter and show different shapes like cocci, rods and other, irregular forms. *Cyanobacteria* are easily identified by their characteristic internal thylakoid membrane stacks (Fig. 1). The different morphotypes resemble subtypes already described from other sponge species by Vacelet (1975), Wilkinson (1978) and Friedrich et al. (1999). Previously described prokaryotic cells with nucleoid-like structures (Fieseler et al. 2004, Fuerst et al. 1998) could also be detected in *I. felix*.

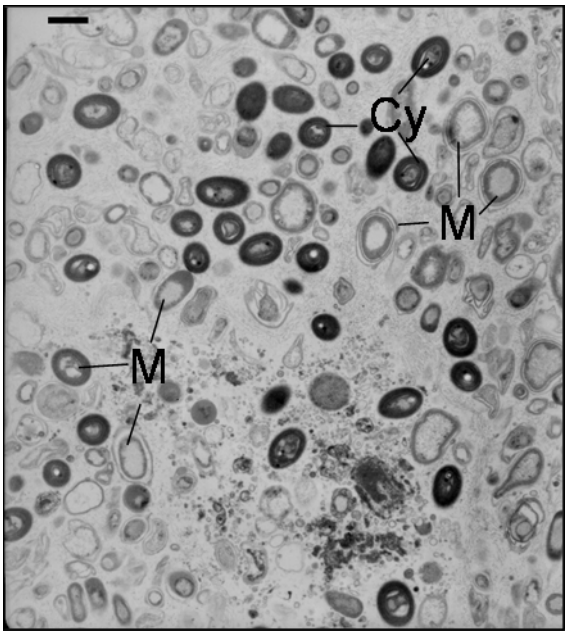

Fig. 1: Transmission electron microscopy of microorganisms in the *I. felix* adult mesohyl. Cy: cyanobacteria, M: microorganisms. Scale bar: 1µm.

I. felix individuals #4 and #5 released parenchymella-type larvae that are a light grey color, about 500µm in length (Fig. 2A) and completely covered with a carpet of small, equally long cilia as seen under the dissecting microscope. Much longer cilia are present at the dark pigmented posterior pole. Fig. 2B gives an overview of a larval cross-section. The ciliary region (C), outer region (OR) and the central region (CR) form three distinct layers. The ciliary region is followed by seemingly two layers of small sponge cells (Fig. 2C, D). In fact, because of diagonal sectioning, the two layers represent the distal and basal part of the same elongated, pear-shaped sponge cells which build an external ciliated epithelium. The distal part (DP) of these cells contains cell organelles like the Golgi apparatus and mitochondria (data not shown). The enlarged basal part (BP) contains the nucleus with condensed chromatin (Fig. 2D). These cells contain large amounts of lipids (pers. communication, A. Ereskovsky, St. Petersburg State University). In the central region of the larvae, electron

transparent amoeboid sponge cells are loosely embedded in the extracellular matrix (Fig. 2E, F). They contain nuclei, phagosomes and many lipids. Visual inspection of at least five different individuals revealed that the outer region of the larvae is almost bacteria-free, whereas the central region of the larvae contains numerous microorganisms. These microorganisms look similar in size, shape and membrane structure to those found in adult samples. Some electron micrographs also show phagosomes containing digested bacterial remnants (Fig. 2E) and bacterial cell division (data not shown).

The juveniles of sponge individuals #4 and #5 showed densely packed electron transparent amoeboid sponge cells. In contrast to the sponge cells of the larvae, juveniles contain less lipids but more phagosomes and vesicles (data not shown). Microorganisms in juveniles are located between sponge cells (Fig. 3A) which show phagosomes indicating microbial phagocytosis. The presence of choanocyte chambers indicates that a functional aquiferous system has already been established (Fig. 3B).

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was used to fingerprint the bacterial community of *I. felix* adult, larval and juvenile stages. Each lane on a gel represents an independent PCR reaction. Banding patterns of the same sample were highly similar, indicating little or no PCR bias between PCRs of the same DNA extraction. Total band numbers were similar for adult #4 (average N=21) and its larvae (average N=22.7) but increased in juveniles (average N=27.5) and the nylon control (average N=27) (Fig. 4A). Cluster analysis of DGGE banding patterns placed the adult and larvae samples together whereas the juvenile clustered with the control sample (Fig. 4B). DGGE band analysis of different larval pools released by the same adult individual revealed highly similar banding patterns that corresponded to that of the adult (Fig. 5A, B). In contrast, comparison of DGGE bands of larvae released by different adult individuals revealed a higher variability (Fig. 6A, B). The similarity of DGGE banding patterns from different larvae pools from the same individual ranged down to 70% whereas that of larvae pools from different individuals was even lower (Fig. 5B, 6B). On the gel in figure 6, some bands were only present (e.g., DGGE band 25) or only absent in a given sample (e.g., DGGE bands 24 and 43 are missing in pool #4). Yet, the majority of bands were still shared between the different samples (e.g., DGGE bands 1, 13, 26, 32).

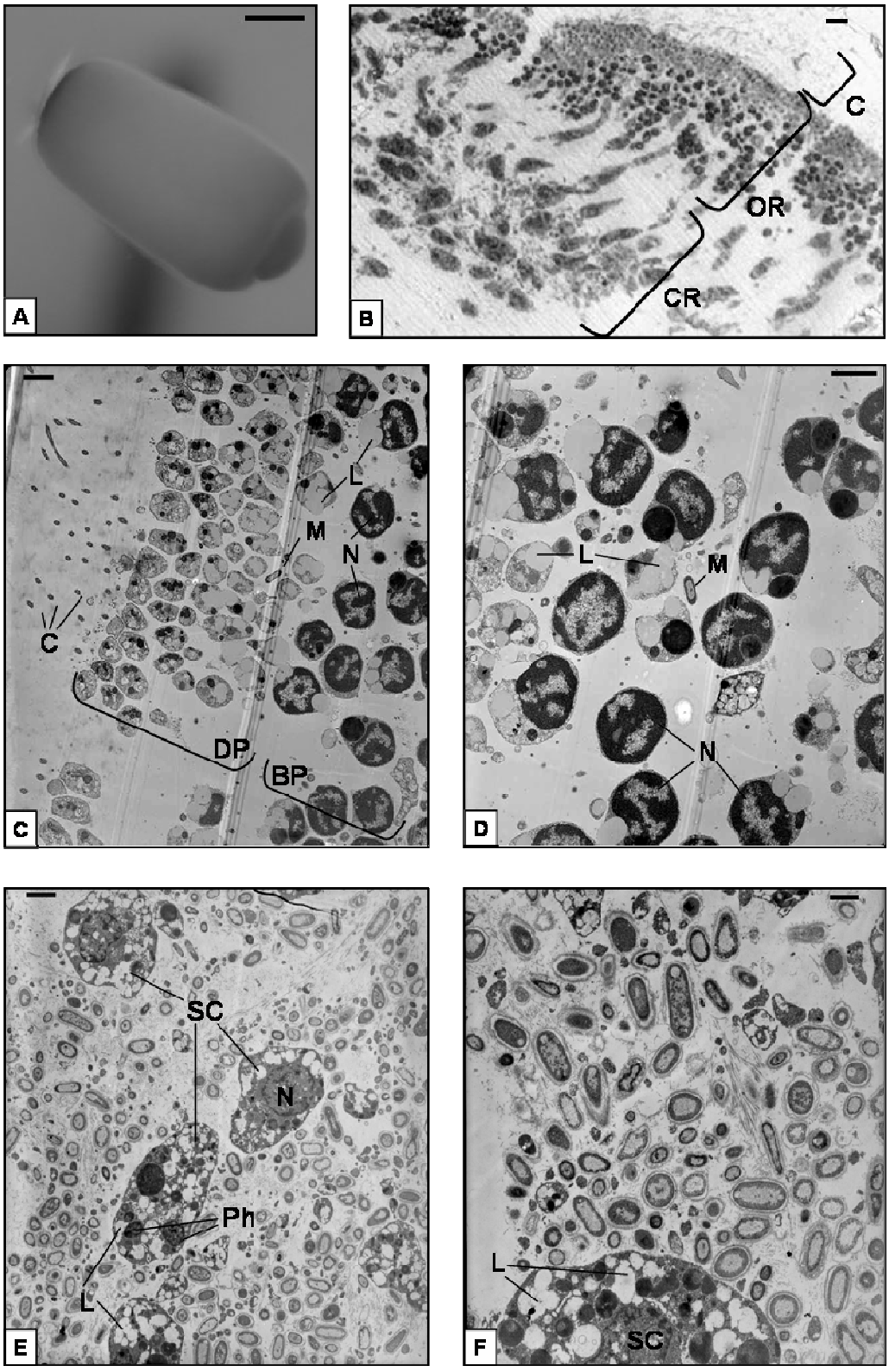

Fig. 2: (A) Dissection microscopy of a parenchymella larva of *I. felix*. (B) Light microscopy of a larva cross-section showing the ciliary, outer and center region. (C, D) Transmission electron microscopy of the ciliary and outer region showing the distal and the basal part of sponge cells. (E, F) Transmission electron microscopy of the central part of the larva with electron transparent sponge cells surrounded by microorganisms. M: microorganisms, BP: basal part, C: ciliary region, CR: central region, DP: distal part, L: lipids, N: nucleus, OR: outer region, Ph: phagosome, SC: sponge cell. Scale bar A (100µm), B (10µm) C, D, E (2µm), F (1µm).

Phylogenetic sequence analysis

In total, 218 sequences were derived from excised DGGE bands. After removal of 54 chimeras (Pintail) and five sequences with undefined sequencing artifacts the remaining 159 sequences were maintained separately according to their source (adult, larvae, juvenile). Each set of sequences was grouped into operational taxonomic units (OTUs) based on a >99% similarity cutoff. One sequence of each of the altogether 112 unique OTUs was chosen and accordingly, 41 adult, 53 larval and 18 juvenile sequences were used for further phylogenetic analysis. The sequences fell into seven different phyla of the domain *Bacteria* (Table 1). One additional sequence cluster was identified that could not be affiliated with any of the publicly available sequence entries (Genbank, May 2006). The adult *I. felix* samples contained members of the phyla *Proteobacteria* (*Alphaproteobacteria*, (n=8), *Gammaproteobacteria* (n=8), *Deltaproteobacteria* (n=2), *Chloroflexi* (n=7), *Acidobacteria* (n=6), *Gemmatimonadetes* (n=5), *Cyanobacteria* (n=3) and members of the cluster of uncertain affiliation (n=2). The DGGE gel derived sequences obtained from *I. felix* larvae represented the *Acidobacteria* (n=13), *Gammaproteobacteria* (n=13), *Alphaproteobacteria* (n=9), *Chloroflexi* (n=6), members of the uncertain cluster (n=5), *Deltaproteobacteria* (n=2), *Actinobacteria* (n=2), *Bacteroidetes* (n=2) and the *Gemmatimonadetes* (n=1). *Cyanobacteria* were absent in larval samples. The phylogenetic diversity of the *I. felix* juveniles revealed members of three phyla which were the *Proteobacteria* (*Alphaproteobacteria* (n=10) and *Gammaproteobacteria* (n=3)), *Actinobacteria* (n=2), *Cyanobacteria* (n=2) and members of the cluster of uncertain affiliation (n=1). It is important to note that the numbers do not reflect the in vivo abundances in the sponge. 63%, 55% and 28% of all sequences obtained from adult, larvae and juvenile samples, respectively, had a sponge-derived sequence as their closest relative (BLAST analysis, as a first approximation).

Clusters of vertically transmitted bacterial groups

Vertically transmitted phylotypes are defined as monophyletic clusters of two or more sequences that were recovered from both, the adult sponge and offspring (larvae and/ or juveniles) of *I. felix* (hereafter termed IF clusters). Altogether 13 monophyletic sequence clusters were identified (Figs. 7, 8) which belonged to four different bacterial phyla and one additional lineage of uncertain affiliation. More than 60% of all *I. felix* derived DGGE sequences fell into these clusters. The in-cluster similarity was above 97% for eight clusters and above 95.5% for 12 clusters. Only the cluster IF-Alpha-2 had a relatively low in-cluster

similarity of 93.9%. Two clusters (IF-Alpha-2, IF-Alpha-4) contained sequences of all three developmental stages (adult, larvae and juveniles).

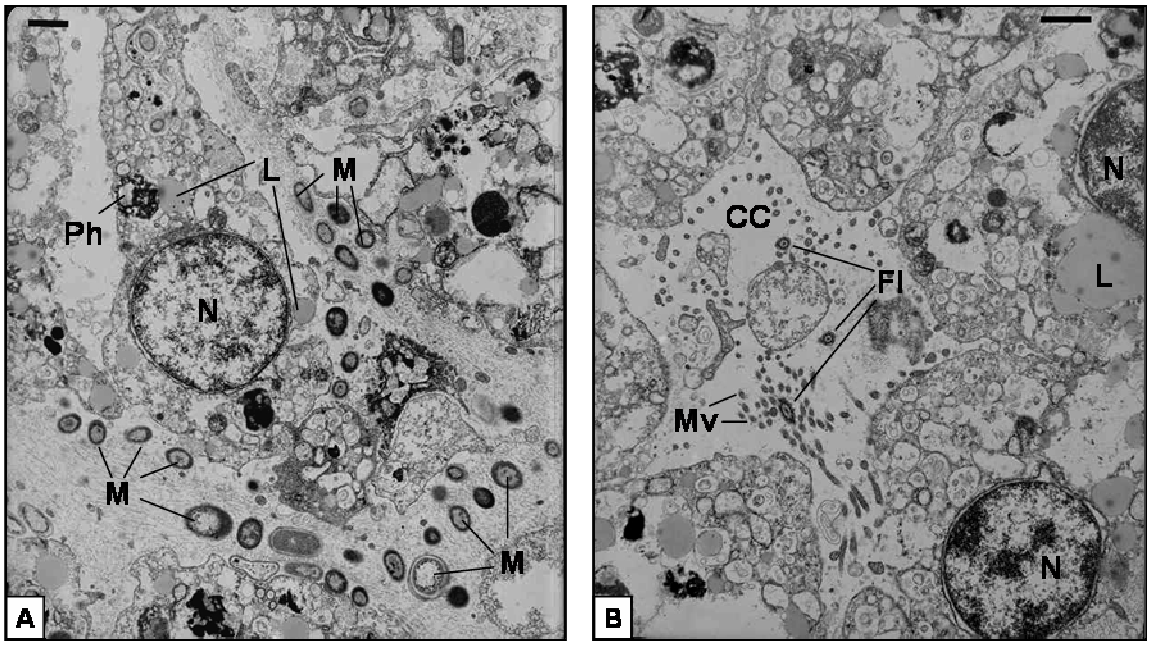

Fig. 3: (A) Transmission electron microscopy of microorganisms in the *I. felix* juvenile mesohyl. (B) Transmission electron microscopy of a choanocyte chamber in a juvenile. M: microorganisms, CC: choanocyte chamber, Fl: flagella, L: lipids, Mv: microvilli, N: nucleus, Ph: phagosome. Scale bar: 1µm.

V. Discussion

Vertical transmission, the passage of microbial symbionts to the next host generation through the reproductive cell lines, is a hallmark of evolutionarily ancient symbioses. Vertical transmission has been documented in terrestrial insects such as ants (Sauer et al. 2002) and aphids (Baumann et al. 1995) as well as in marine invertebrates such as bryozoans (Lim and Haygood 2004) and bivalves (Cary and Giovannoni 1993, Sipe et al. 2000). Since the passage of symbionts via the reproductive stages is highly selective, this process frequently leads to cospeciation between the host and symbiotic lineages resulting in congruent phylogenetic trees (Peek et al. 1998). The presence of bacteria in the reproductive stages has also been demonstrated in representatives of all three sponge classes by electron microscopy suggesting that vertical transmission is a widespread mechanism in this phylum (Ereskovsky et al. 2005 and references cited therein). In comparison to the well-studied symbioses mentioned above, the microbial associations of sponges are different in several respects: Rather than the known

one-(few)-symbiont-one-host types of associations, the microbial consortia of sponges are exceedingly complex containing sponge-specific representatives of at least eight different bacterial phyla and one archaeal phylum (Hentschel et al. 2006). Secondly, the microbial biomass within the bacteriosponges (high-microbial-abundance-sponges) is massive contributing up to 40-60% of the animal's biomass (Vacelet 1975, Wilkinson 1978). To our knowledge, no other animal phylum tolerates such amounts of internal, freely dispersed microorganisms. The enormous microbial biomass in vertebrate intestines is also located internally but is contained within specialized organs, such as the rumen. Thirdly, because of the characteristic anatomy of sponges, there are no physical barriers such as organs or tissues that separate the sites of reproduction from the mesohyl microbiota. In other symbioses, symbionts are frequently contained in specialized cells (bacteriocytes) or modified host organs (i.e., the trophosome of the tubeworm *Riftia pachyptila* and the glands of Deshayes of the shipworm *Bankia setacea*). It was therefore of interest to document the passage of microorganisms via the reproductive stages of a Caribbean demosponge and to phylogenetically characterize the bacterial consortia within them. A combination of visual (electron microscopy) and molecular techniques (DGGE) was used towards this goal.

Electron microscopy studies provided first insights into the presence of microorganisms within the larvae of *I. felix* (Fig. 2). High amounts of morphologically diverse microorganisms were located extracellularly in the central part of the larvae while the outer rim appeared almost bacteria-free. This is in agreement with the microscopic description of the Mediterranean species *Ircinia oros* which also contained numerous bacteria in the inner part of the larvae but not in the peripheral region (Ereskovsky and Tokina 2004). In general, the larvae of both *Ircinia* species have a similar morphology including dark pigmented cells and a ring of elongated cilia at the posterior pole which might be important in the response to external stimuli during the planktonic life stage (Leys and Degnan 2001). Both *Ircinia* species have a ciliated epithelium consisting of densely packed sponge cells. This pear-shaped cell form and the location of the nucleus in the basal part are unusual as the nucleus is located more distally in most parenchymella larvae (Ereskovsky and Tokina 2004). The central part of the *I. felix* larvae contains only one loosely associated cell type, which has been identified as archaeocytes in *Ircinia oros* (Ereskovsky and Tokina 2004) and other dictyoceratid species (Kaye and Reiswig 1991). Finally, both *Ircinia* larvae contain unusually high amounts of lipids which is consistent with the lecithotrophic (non-feeding) nature of these larvae. This

reserve substance may allow for a long pelagic life phase and would increase the chance of survival during metamorphosis.

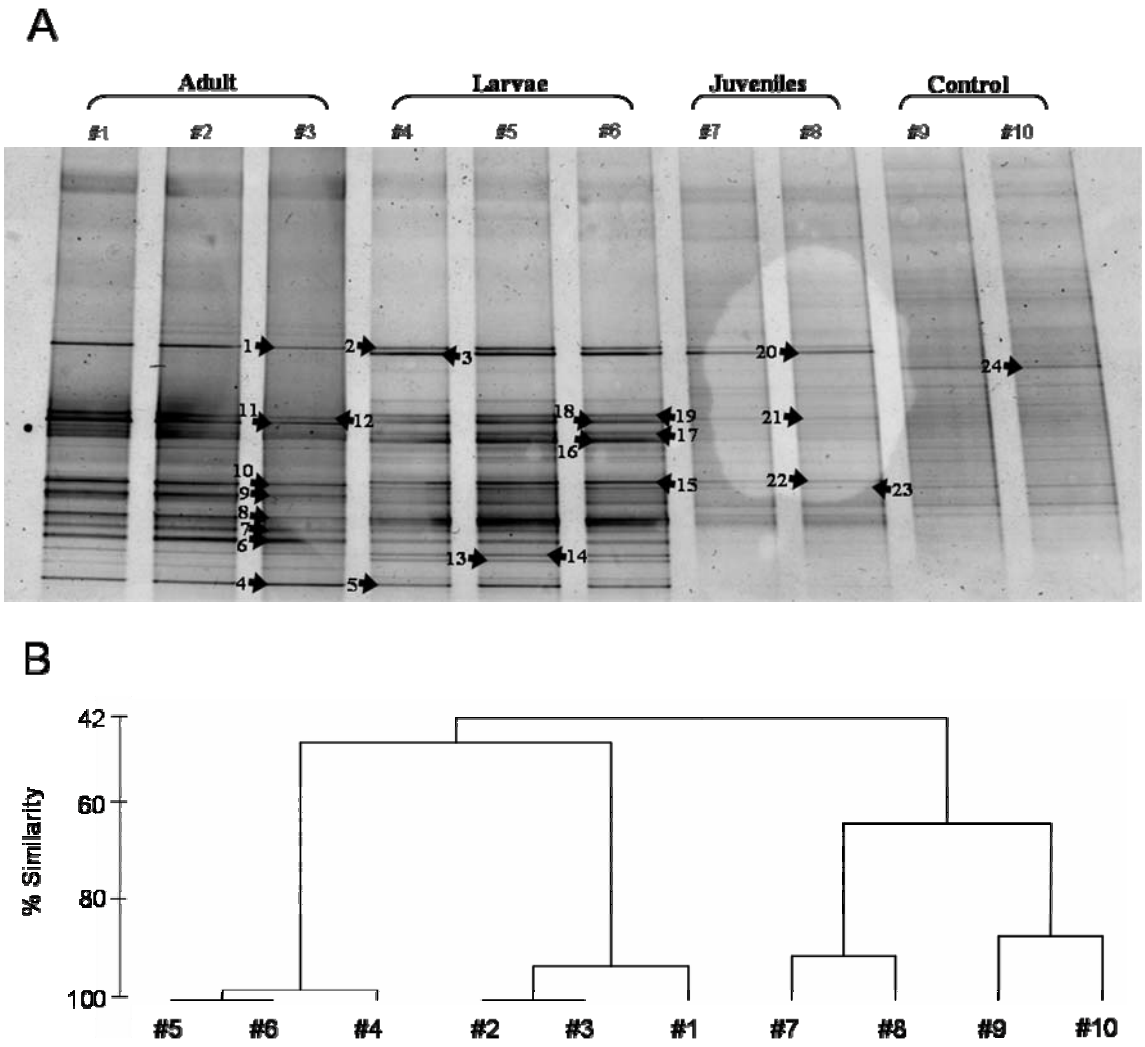

Fig. 4: (A) Denaturing gradient gel electrophoresis (Gel A) of the adult, larvae and juvenile samples (individual #4). Three independent PCR reactions were run each for adult and larvae samples and two each for the juvenile and the control sample. A piece of nylon without sponge tissue taken after the settlement experiment was used as a control. Arrows indicate excised and sequenced bands. (B) Dendrogram showing percentage similarity of banding patterns. Numbers correspond to lanes in the gel.

The bacterial community profile of the adult *I. felix* sponge strikingly resembles that of other high microbial abundance sponges that have been subject to molecular microbial diversity analysis (i.e. Hentschel et al. 2002, Schirmer et al. 2005, Taylor et al. 2004, Thoms et al. 2003, Webster et al. 2001). *I. felix* contained sponge-specific sequences from seven of the eight previously reported bacterial phyla (Table 1). These are the *Proteobacteria* (*Alpha-*, *Gamma-*, *Deltaproteobacteria*) *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi* and

Cyanobacteria. Members of two sequence clusters previously reported as 'uncertain affiliation-I and -II' (Hentschel et al. 2002), now known to belong to the phylum *Gemmatimonadetes* (Zhang et al. 2003, this study) were also recovered from *I. felix*. Furthermore, a deeply-routing sequence cluster was identified in this study that could not be affiliated with any of the known sequences available in the public libraries. The closest relatives are an unidentified clone from the Mediterranean sponge *Aplysina cavernicola* (Genbank accession number AY180080, >97% similarity) (Thoms et al. 2003) and an unidentified activated sludge clone (Genbank accession number AF097803, 94.4% similarity) (Fig. 8). This sequence cluster might represent a novel clade of sponge-specific bacteria. Additionally, a gammaproteobacterial cluster (IF-Gamma-3) is noteworthy as it forms a coherent clade with a number of 16S rRNA gene sequences from chemoautotrophic symbionts of deep-sea invertebrates including gastropods, bivalves and tubeworms.

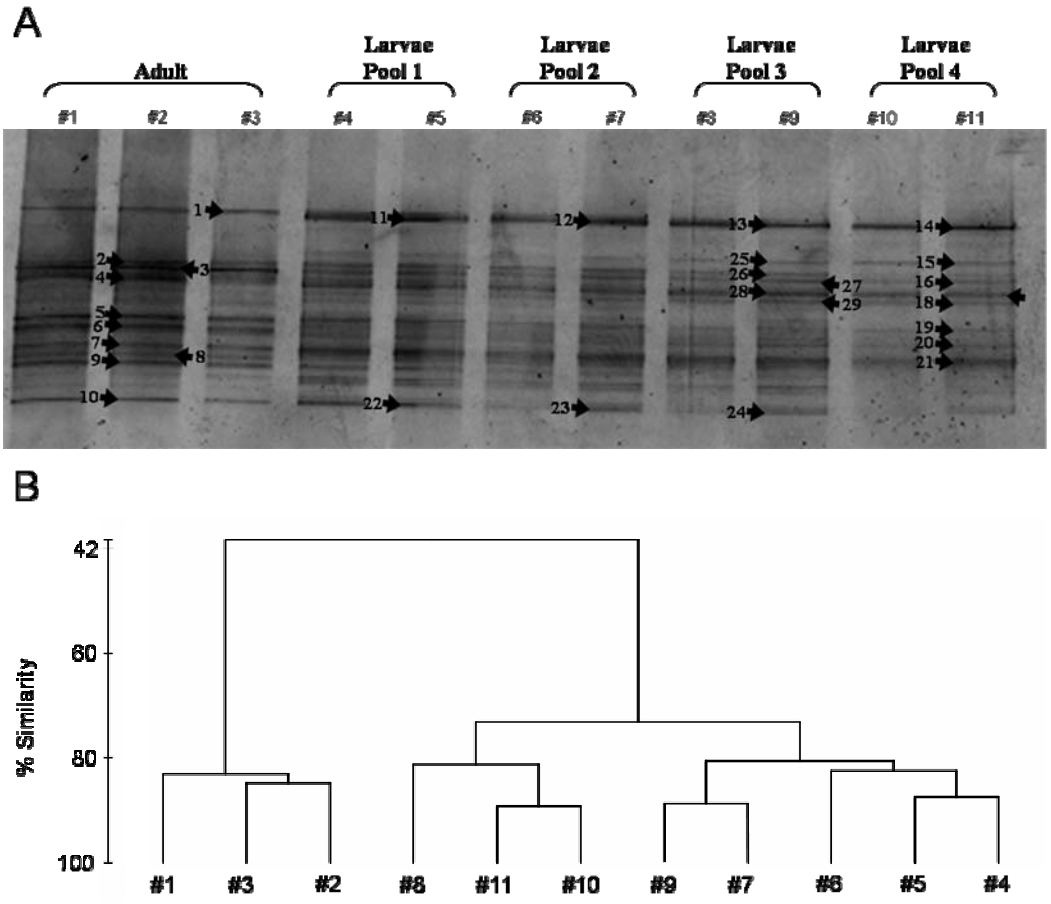

Fig. 5: (A) Denaturing gradient gel electrophoresis (Gel E) of *I. felix* adult #4 compared to different larvae pools released by the same adult specimen. Three independent PCR reactions were run for adult, and two each for pooled larvae. Arrows indicate excised and sequenced bands. (B) Dendrogram showing percentage similarity of banding patterns. Numbers correspond to lanes in the gel.

Interestingly, several previously reported sponge-specific clusters (Hentschel et al. 2002) could not be detected in this study (Figs 7, 8). These are the 'Nitrospira-I' cluster of the phylum *Nitrospira* that contains exclusively nitrite-oxidizing bacteria. The gammaproteobacterial 'Gamma-I' cluster that is most closely related to the ammonia-oxidizing *Nitrosococcus* sp. was also not identified. If the coordinated metabolism of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria is responsible for the process of nitrification in sponges, then the conspicuous absence of both clades would suggest that eubacterial nitrification is not an important process in *I. felix*. Nevertheless, nitrification mediated by Archaea, such as for example the *Cenarchaeum symbiosum* clade of sponge symbionts, remains a distinct possibility (Hallam et al. 2006, Preston et al. 1996). Members of the recently discovered candidate phylum '*Poribacteria*' and of the domain *Archaea* would not have been expected in this study because of mismatches in the PCR primer regions. Several lineages have so far exclusively been found in *I. felix*. These are the IF-Alpha-3 cluster related to a coral associated clone (Genbank accession number DQ200432), the IF-Alpha-4 cluster related to *Rhodovulum* sp. (Genbank accession number AM180953) and the IF-Acido-2 cluster related to acidobacterial clones from soil (Genbank accession numbers AY921986, AB240276, AY922161). Whether these lineages are specific to the sponge *I. felix* remains to be seen as more sequences from other *Ircinia* sponges become available.

The bacterial diversity of the *I. felix* larvae is comparable to that of the adult sponge with respect to the DGGE band numbers, patterns and the phylotypes recovered (Figs. 4, 7, 8). The banding patterns of different larvae pools from the same individual were more similar than those from different individuals (Figs. 5A, 6A), a fact that is also reflected in the cluster analysis (Figs. 5B, 6B). Deciphering the degree of fine scale intra-species variation poses a challenge for further studies. Sequencing and phylogenetic analysis of the DGGE bands showed the presence of all previously identified, sponge-specific bacterial phyla (Hentschel et al. 2006, Hill et al. 2004, Imhoff and Stöhr 2003) in the larvae with the exception of the *Cyanobacteria*. Since DGGE bands of the appropriate migration distance were also identified in the larval sample (Fig. 4), it is possible that the cyanobacterial phylotypes are present in the larvae but that the corresponding bands were not chosen for sequencing. Alternatively, since the larvae are brooded in the mesohyl and the *Cyanobacteria* are predominantly found on the outer surface, they might be incorporated into the larvae less efficiently. In fact, Usher has shown that only 25% of all larvae from *Chondrilla australiensis* contained *Cyanobacteria* (Usher et al. 2001).

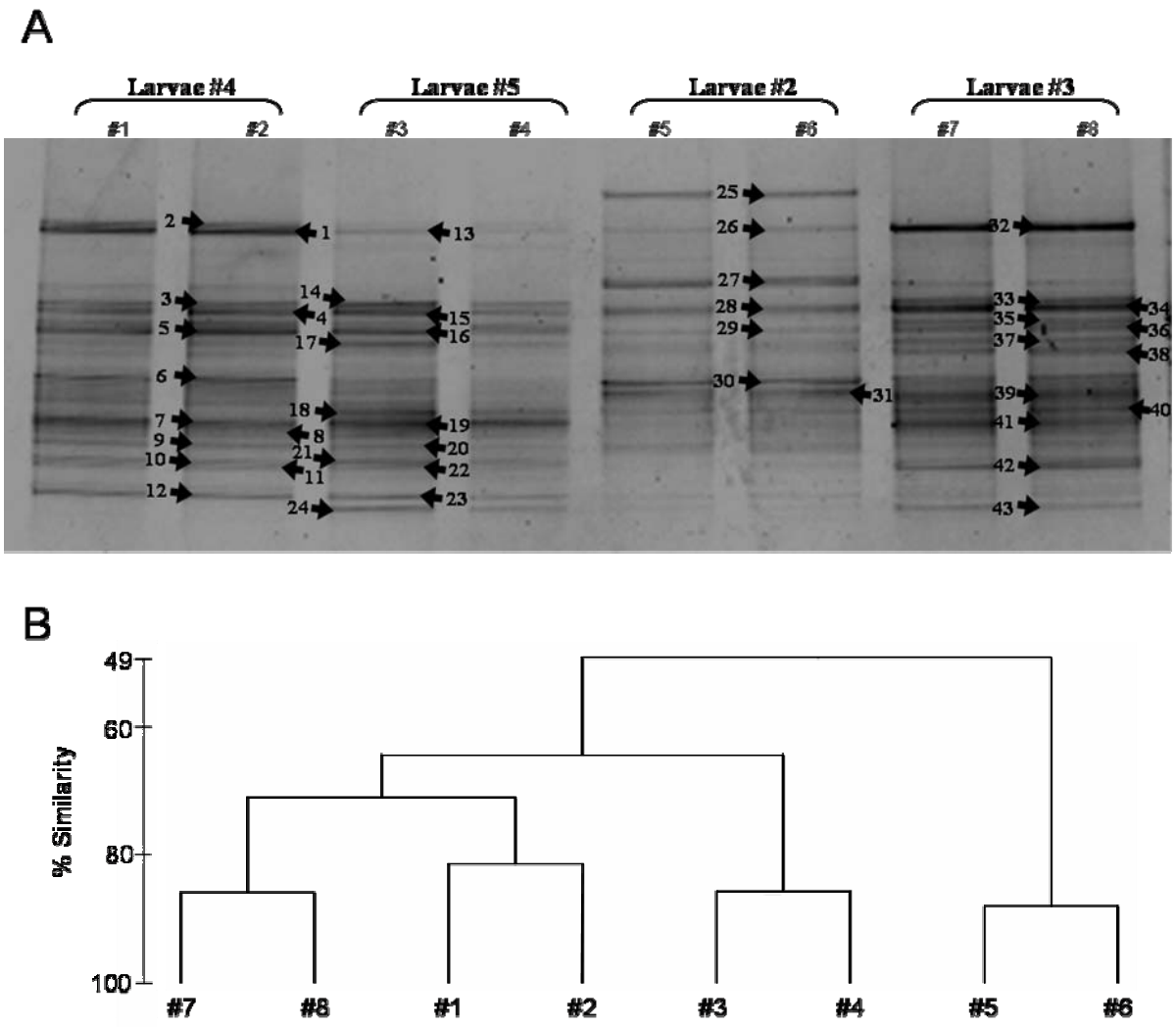

Fig. 6: (A) Denaturing gradient gel electrophoresis (Gel F) of four larvae pools released by four different adult specimens (#2, 3, 4, 5). Two independent PCR reactions were run each. Arrows indicate excised and sequenced bands. (B) Dendrogram showing percentage similarity of banding patterns. Numbers correspond to lanes in the gel.

The DGGE banding pattern of the *I. felix* juvenile sample contained more bands than the corresponding adult and larvae samples. The dendrograms clustered the adult and larval sample together while the juvenile formed one cluster with the control. The higher number of DGGE bands is probably an artifact from the extraction protocol as the newly grown sponges were still attached to the nylon substrate at the time of DNA extraction. Hence, the DGGE banding pattern is a mixture of sponge-specific phylotypes and colonizers from seawater. Altogether, representatives of three bacterial phyla could be identified including sponge-specific lineages of the phyla *Actinobacteria*, *Alphaproteobacteria* and the clade of uncertain affiliation. The reduced bacterial diversity might be due to methodological constraints as more than twice as many sequences were gained from adult samples (n=41) and from larvae

(n=53) than from juveniles (n=18) which showed a generally more faint banding pattern. If more sequences would have been obtained from juveniles, the number of identified phyla that contain sponge-specific representatives might have been higher.

Table 1: Phylogenetic diversity of bacteria associated with adult, larvae and juvenile *I. felix*.

Bacterial phylum	Adult	Larvae	Juveniles
Acidobacteria	+	+	–
Actinobacteria	–	+	+
Bacteroidetes	–	+	–
Chloroflexi	+	+	–
Cyanobacteria	+	–	+
Gemmatimonadetes	+	+	–
Proteobacteria (Alpha)	+	+	+
Proteobacteria (Gamma)	+	+	+
Proteobacteria (Delta)	+	+	–
Uncertain affiliation	+	+	+

+: present; –: absent

Alternatively, the reduced diversity in juveniles may be correlated to the feeding behaviour of sponge larvae. Free-swimming larvae are unable to take up food particles from the water column (Jaeckle 1995). If the internal sponge symbionts would serve as food during the non-feeding planktonic phase, then the microbial community within the larvae would be naturally reduced. This hypothesis is supported by electron microscopical observations that show evidence of phagocytosis in the larvae (Fig. 2E). Further experiments focussing on the juvenile stages are necessary to determine whether the microbial diversity is truly reduced or whether the sponge-specific lineages are present in the larvae, albeit below the limit of detection. Conceivably, it would need only a single bacterium of each lineage to inoculate the newly grown sponge.

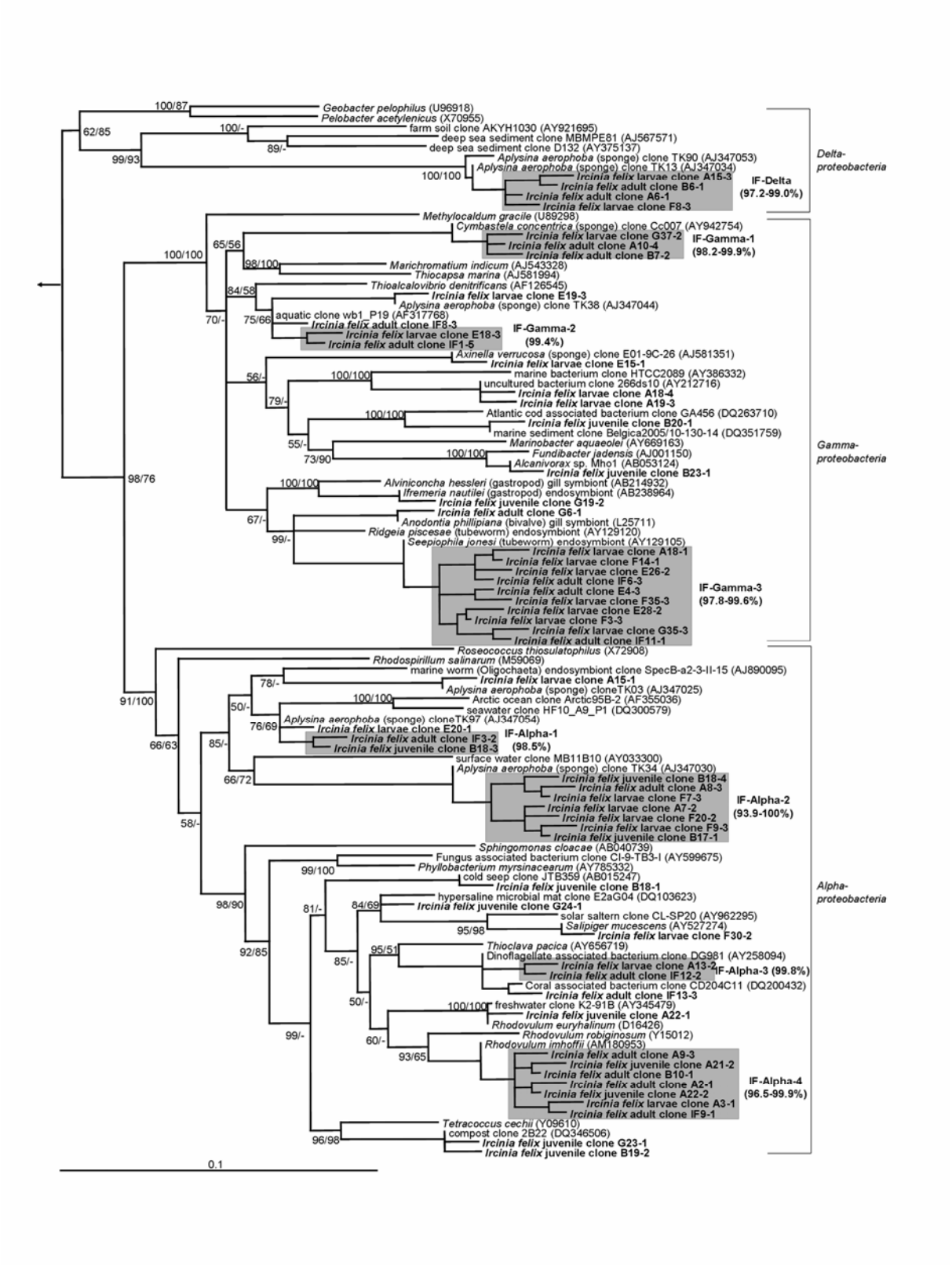

Fig. 7: Phylogenetic distance tree calculated with 16S rDNA proteobacterial DGGE-sequences recovered from *I. felix*. Neighbor-joining and maximum parsimony (100 pseudoreplicates) bootstrap values are provided. Sequences obtained in this study are in bold. The clones are coded as follows: DGGE gel (capital letter), excised band (first number), clone (second number). Grey boxes depict monophyletic clusters of sequences that originated from both, adult and offspring (larvae and/ or juveniles) of *I. felix*. Scale bar indicates 10% divergence. Arrow, to outgroup (*Geothrix fermentans* U41563, *Holophaga foetidae* X77215, *Acidobacterium capsulatum* D26171).

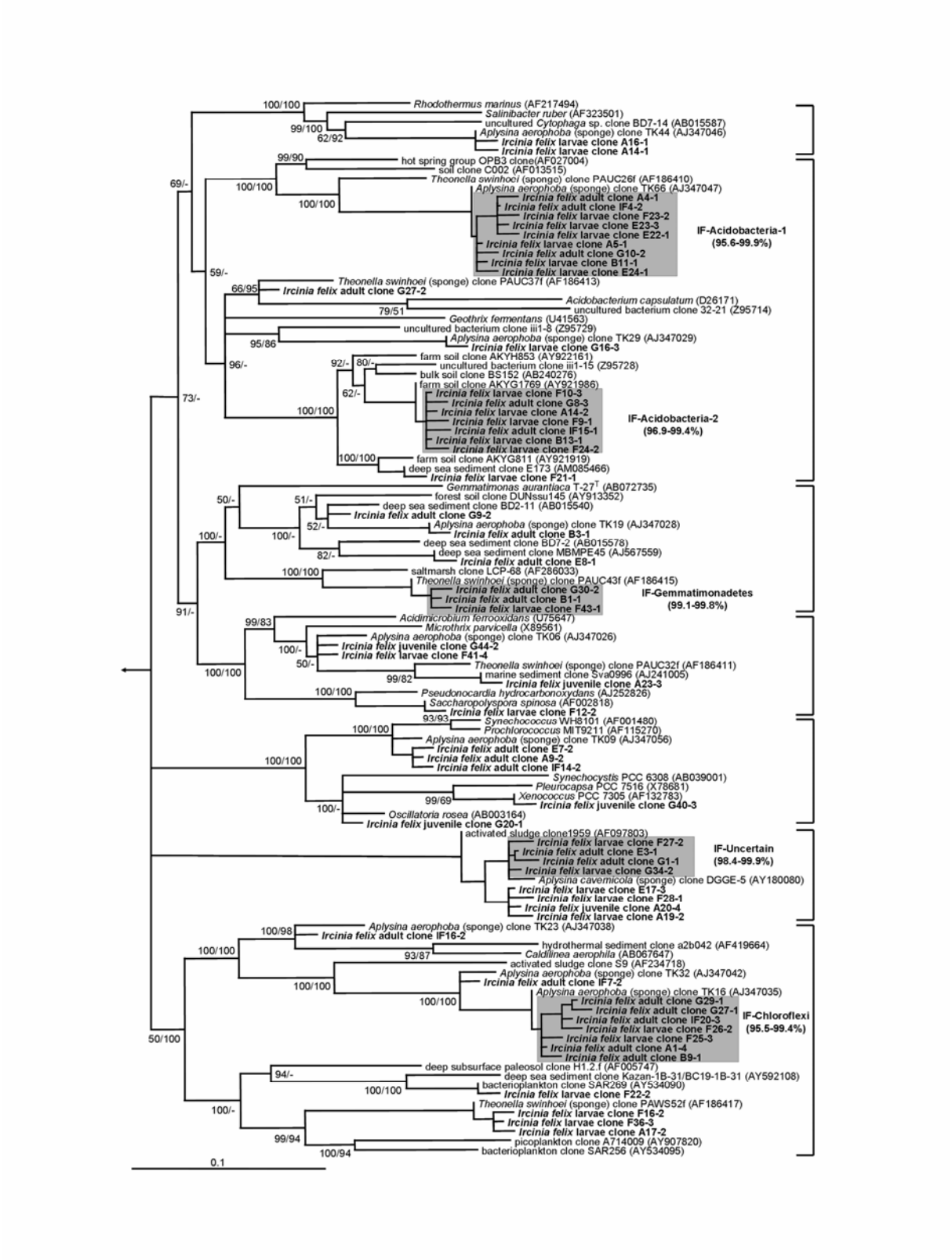

Fig. 8: Phylogenetic distance tree calculated with 16S rDNA bacterial DGGE-sequences recovered from *I. felix*. Neighbor-joining and maximum parsimony (100 pseudoreplicates) bootstrap values are provided. Sequences obtained in this study are in bold. The clone labels are coded as follows: DGGE gel (capital letter), excised band (first number), clone (second number). Grey boxes depict monophyletic clusters of sequences that originated from both, adult and offspring (larvae and/ or juveniles) of *I. felix*. Scale bar indicates 10% divergence. Arrow, to outgroup (*Pyrobaculum calidiformis* AB078332, *Sulfolobus metallicus* D85519, *Desulfurococcus mobilis* M36747).

In summary, it could be shown that in the sponge *I. felix*, the entire microbial consortium rather than individual phylogenetic lineages are passed on to the next generation via the reproductive stages. Accordingly, vertical transmission is specific in that the microorganisms of *I. felix*, but not those from seawater, are passed on, but unselective in that there appears to be no differentiation between individual sponge-specific lineages. These data are congruent with those by Sharp et al. (Sharp et al. 2007) who reported on vertical transmission of similarly complex phylogenetic lineages throughout embryonic development of *Corticium* sp.. This passage of diverse microorganisms to the next generation is probably due to the characteristic anatomy of sponges where the reproductive elements are exposed to microorganisms in the mesohyl, whereas in higher animals, the reproductive elements are contained in specialized, bacteria-free reproductive organs. In conclusion, the ancient and wide spread mechanism of vertical transmission is clearly important for the formation and maintenance of the phylogenetically complex yet highly sponge-specific microbial communities of many marine demosponges.

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Chapter Five

Marine sponges as models for commensal microbe-host interactions

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I. Abstract

In nature, multiple-species rather than single-species microbial associations with plant or animal (including human) hosts are the rule more than the exception. Prominent examples are the microbial consortia of vertebrate intestines and cattle rumen. As many demosponges are associated with enormous amounts of microorganisms, contributing up to 40 - 60% of the sponge biomass, they are excellent models for marine multi-species, microbe-host associations. Representatives of at least eight different phyla, many of which contain few or no cultivated representatives, have been identified as specific members of the sponge-associated microbiota. Recent studies show that vertical transmission of symbionts through the larval stages rather than horizontal acquisition from seawater appears to be an important mechanism by which the complex and possibly ancient microbial consortia of sponges are formed.

II. Introduction

Symbioses in the classical sense of de Bary (1879) are nowadays defined as mutualistic, commensal or pathogenic bacteria-host interactions (Douglas, 1994; Hentschel et al., 2000; 2002). Mutualistic bacteria-host interactions are balanced relationships with reciprocal benefit. Commensal interactions imply the coexistence of at least two different organisms without detriment, but possibly with a benefit to one partner. In pathogenic interactions one partner benefits to the detriment of the other causing cell or tissue damage or even death of the organism. The interactions between bacteria and their hosts are not static, rather they evolve continuously to maintain equilibrium. While mutual symbioses are frequently ancient in time, pathogenic ones seem to have evolved more recently (Hentschel et al., 2000; Steinert et al., 2000).

Many of the well known symbioses come from the marine world (Table 1). They are typically based on primary metabolism where symbionts provide access to autotrophically fixed nutrients (carbon or nitrogen) in a nutrient-poor environment. For example, through the acquisition of photoautotrophic symbionts, corals can build large reefs in tropical oligotrophic waters. On the other hand, a number of bacterial and fungal pathogens have been identified as the causative agents of coral bleaching (Rosenberg and Ben-Haim, 2002). Through

incorporation of chemoautotrophic symbionts, worms, clams and mussels can populate deep-sea hydrothermal vents, cold seeps, seawage outfalls and anoxic sediments (Fischer, 1990). Through the aid of cellulose-digesting symbionts, shipboring bivalves are able to establish an existence in driftwoods (Distel, 2003). Other marine symbioses are based on secondary metabolism where microbial symbionts provide chemical defenses for their hosts as it appears to be the case for bryozoans and possibly also for sponges (Haygood et al., 1999). An unusual behavioural symbiosis is represented by the light-producing *Vibrio fischeri* symbionts of sepiolid squids that probably serve to deter predators at night (Nyholm and McFall-Ngai, 2004). By providing essential functions (i.e., nutrient acquisition, chemical defense, behavioural mimicry) the respective animal hosts can populate new niches that would otherwise be inaccessible. This appears to be an important ecological advantage in the homogenous ecosystem of the ocean where physical barriers and geographic structures are scarce.

Most of the established marine symbiosis models are based on the interaction of a given host or host lineage with one or few specific symbionts. Among the invertebrate hosts, certain oligochaetes are unusual in that they are specifically associated with at least four different symbiotic lineages. The metabolically different microorganisms presumably allow the worm to migrate through oxidizing and reducing sediments (Woyke et al., 2006). Among the microbial symbionts, *Symbiodinium* algae are unusual in that a single symbiotic lineage (clade C) populates many dozens of different Great Barrier Reef corals (LaJeunesse et al., 2003).

In comparison to the well-studied symbioses mentioned above, the microbial associations of sponges are different in several respects: Rather than the known one-(few)-symbiont-one-host types of associations, the microbial consortia of sponges are exceedingly complex containing sponge-specific representatives of at least eight different prokaryotic (bacterial and archaeal) phyla. To our knowledge, no other animal phylum tolerates such amounts of internal, freely dispersed microorganisms. Most other symbionts are housed in specific, symbiont-bearing cell layers (gastrodermis of corals and sea anemonies) or organs (gland of Deshayes in shipworms, light organs of squids, trophosome tissue of vestimentiferan worms) or even in specialized cells, termed bacteriocytes. Because sponges are sufficiently distinct from the other established marine symbioses, they are worthwhile models to study marine commensal bacteria-host interactions.

Table 1: Selected marine symbiosis models.

Animal phylum	**Invertebrate host**	**Microbial symbiont**	**Type of interaction**	**Proposed function of symbiont**
Porifera	Sponges (ie., *Aplysina aerophoba, Theonella swinhoei, Discodermia dissoluta, Rhopaloeides odorabile, Cymbastela concentrica*)	Sponge-specific lineages within the domains Bacteria [*Alpha-, Gamma-, Deltaproteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Gemmatimonadetes, Nitrospira* and *cand. phylum* Poribacteria] and Archaea (*Crenarchaeota*)	commensal	unknown
Cnidaria	Corals (i.e., *Acropora tenuis*, *Stylophora pistillata*) and sea anemonies (i.e., *Anthopleura elegantissima*)	Dinoflagellate algae (*Symbiodinium* sp.)	beneficial	Photosynthetic symbionts provide CO_2 to the coral host
Cnidaria	Corals (various)	Gammaproteobacteria (*Vibrio* sp.)	pathogenic	Symbiont expulsion and bleaching
Annelida	Oligochaetes (*Olavius* sp., *Inanidrilus* sp.)	Gamma- and Deltaproteobacteria	beneficial	Chemoautotrophic symbionts provide host with multiple sources of nutrition and probably recycle worm waste products
Vestimentifera	Worms (*Riftia pachyptila*)	Gammaproteobacteria	beneficial	Chemoautotrophic symbionts provide CO_2 to the gutless host
Mollusca	Shipworms (Teredinidae i.e., *Lyrodus pedicellatus*, *Bankia setacea, Xylophaga atlantica*)	Gammaproteobacteria (i.e., *Teredinibacter turnerae*)	beneficial	Heterotrophic symbionts provide enzymes for cellulose digestion
Mollusca	Squids (*Euprymna scolopes*)	Gammaproteobacteria (*Vibrio fischeri*)	beneficial	Bioluminescent symbionts provide light to deter predators at night
Mollusca	Sea Slugs (i.e., *Elysia chlorotica*)	Heterokont algae (*Vaucheria litorea*)	beneficial	Algae chloroplasts provide photoautotrophically fixed carbon to animal host.
Mollusca	Bivalves and Mussels (ie., *Lucinoma* sp. *Bathymodiolus* sp.)	Gammaproteobacteria	beneficial	Chemoautotrophic and methanotrophic symbionts provide CO_2 to the gutless host

Animal phylum	Invertebrate host	Microbial symbiont	Type of interaction	Proposed function of symbiont
Bryozoa	Moss animals (i.e., *Bugula neritina*, *B. simplex*)	Gammaproteobacteria (*Candidatus* Endobugula glebosa)	beneficial	Heterotrophic symbionts provide secondary metabolites (polyketides) for chemical defense
Vertebrata	Fish (i.e., *Photoblepharon palpebratus* ('Eyelight fish')	Gammaproteobacteria (*Photobacterium* sp.)	beneficial	Bioluminescent symbionts provide light to deter predators and attract prey and are used for communication with mates

III. The players: Sponges as animal hosts

The phylum sponges (*Porifera*) forms one of the deepest radiations of the Metazoa with a fossil record dating back more than 580 million years (Li et al., 1998). Well over 1000 sponge fossils have been described within 15 genera and 30 species in Precambrian rock deposits suggesting an early radiation of this phylum. The phylum Porifera is divided into three classes, the Calcarea (calcareous sponges), the Hexactinellida (glass sponges) and the Demospongiae which contain more than 90% of the sponges living today. An estimated 13,000 living sponge species are found on tropical reefs but also with increasing latitudes and even in freshwater lakes and streams. Sponges are known for their colorful appearance and the morphological plasticity that ranges from encrusting layers several millimeters in height to massive tubular sponges of > 1 meter in size.

Sponges are diploblast metazoans that lack true tissues or organs. In spite of their simple organisation, genome sequencing has revealed genes that are highly similar to those of vertebrates (Müller et al., 2001). As sessile filter-feeders they pump large volumes of water through a specialized canal system, termed the aquiferous system. The filtration capacities of sponges are remarkably efficient amounting to many thousands of liters kg^{-1} day^{-1}. While typical seawater contains 10^5- 10^6 bacteria ml^{-1}, the seawater expelled from the sponge is essentially sterile (Wehrl et al., in press). Food particles such as unicellular algae and bacteria are retained in the choanocyte chambers and translocated into the mesohyl interior where they are rapidly digested. The mesohyl serves as a scaffold that is made up of extracellular matrix

and constitutes much of the sponge body. Single, amoeboid sponge cells, termed archaeocytes, move freely through the mesohyl matrix and digest food particles by phagocytosis.

IV. The players: Microbial consortia as symbionts

Many species of the demosponges are known to contain large amounts of bacteria within their tissues which may contribute up to 40 - 60% of the tissue volume (for reviews, see Lee et al., 2001; Hill 2004; Hentschel et al., 2006). These sponges are called 'bacteriosponges' or 'high microbial abundance sponges' (Vacelet and Donadey, 1977; Wilkinson 1978; Hentschel et al., 2003b; Schmitt et al., submitted). Microbial concentrations amount to 10^8 to 10^{10} microbial cells ml^{-1} sponge extract exceeding the concentrations of seawater by two to five orders of magnitude. The vast majority of microorganisms are located extracellularly within the mesohyl matrix. Microorganisms are also found within vacuoles of archaeocytes where they appear in various stages of digestion. In some cases, microorganisms are located within the nuclei of certain sponge cells. The sponge surfaces, the canal system and choanocyte chambers are noticeably free of microorganisms. There are other sponge species that coexist in the same habitat whose mesohyl is essentially free of microorganisms. They are referred to as 'low microbial abundance sponges' (Vacelet and Donadey, 1977; Wilkinson 1978; Hentschel et al., 2003b; Schmitt et al., submitted). The microbiota of low-microbial-abundance-sponges reflects that of seawater, both in numbers and in phylogenetic composition. The reasons why some sponges are hosts to large intrinsic microbiota while others are virtually devoid of microorganisms are currently unknown.

About a dozen studies have addressed the phylogenetic diversity of microbial communities associated with marine bacteriosponges using 16S rRNA gene library construction and other 16S rRNA gene based techniques (i.e., Webster et al., 2001b, Hentschel et al., 2002, Schirmer et al., 2005, Taylor et al., 2005). The sponges of these studies are taxonomically only distantly related, have geographically non-overlapping distribution patterns and contain host-specific secondary metabolite profiles. In spite of these differences, the sponges contain a uniform microbial signature (Hentschel et al. 2002). Particularly representatives of the phyla *Acidobacteria*, *Actinobacteria, Bacteroidetes* (formerly CFB group or *Cytophagales*), *Chloroflexi* (formerly green-non-sulfur bacteria), *Cyanobacteria*, *Proteobacteria* (*Alpha*-,

Gamma-, *Delta-*) and *Nitrospira* were frequently recovered by this approach (Fig. 1). The Harbor Branch Marine Microbial Database (HBMMD) (http://www.hboi.edu/dbmr/dbmr_hbmmd.html) represents an extensive collection of culturable microorganisms from marine sponges and other deep-water invertebrates (Gunasekera et al., 2005). However, none of the above mentioned, sponge-specific bacteria have been cultivated so far with one noticeable exception. The alphaproteobacterial strain MBIC3368 has been isolated from at least eight different sponge species (Scheuermayer et al. 2006) including adult and larvae samples of the sponge *Mycale laxissima* (Enticknap et al., 2006).

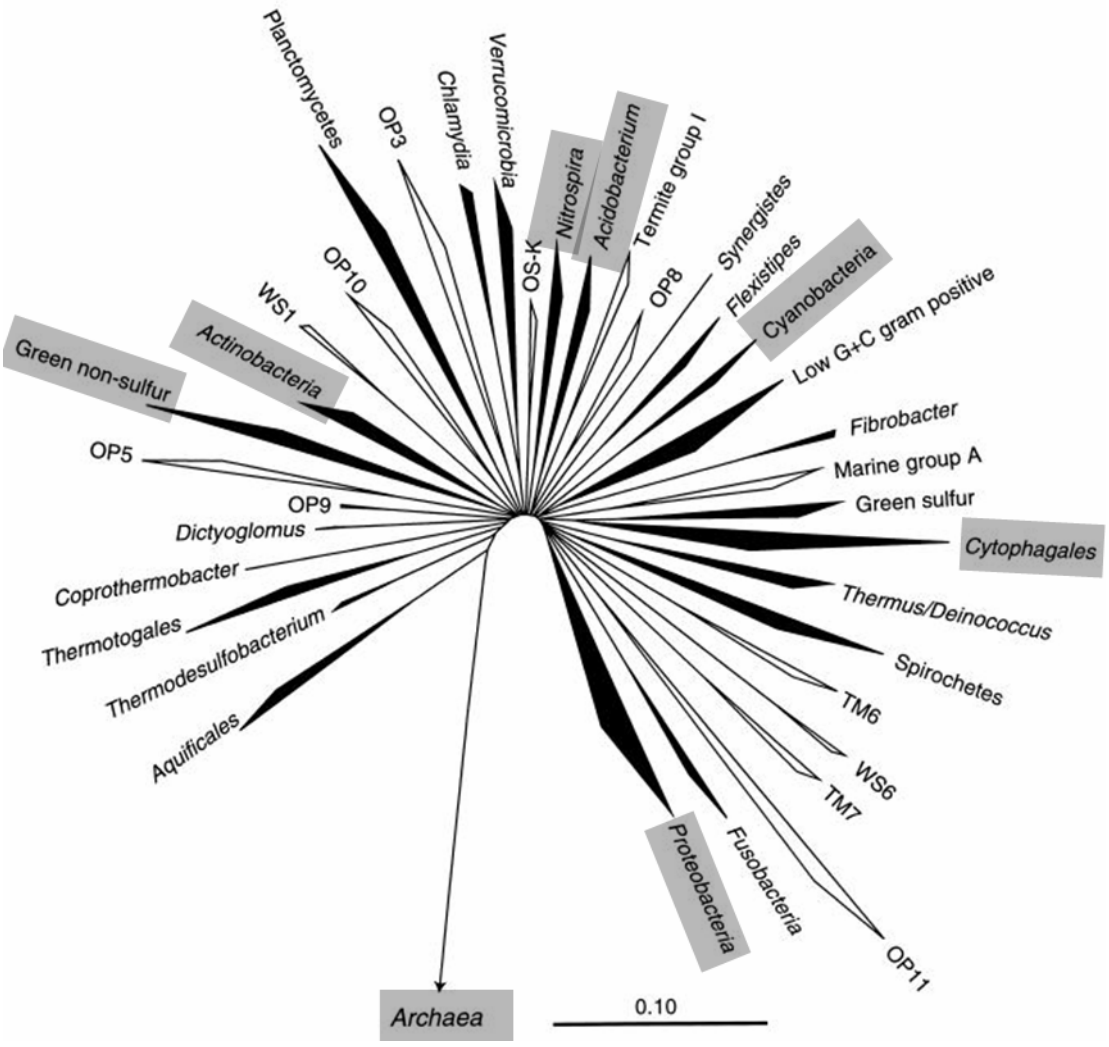

Fig. 1: Phylogenetic tree of the prokaryotes modified after Hugenholtz et al. (1998). Phyla, of which members of the sponge-specific microbiota were identified, are marked with grey boxes. The *Candidatus* phylum 'Poribacteria' falls into the *Planctomycetes-Verrucomicrobia-Chlamydiae* superphylum (Wagner and Horn, 2006). The green non sulfur bacteria are now termed *Chloroflexi*. Most lineages were also identified by DGGE in the reproductive stages of *I. felix* with the exception of the *Nitrospira* and *Cyanobacteria* (Schmitt et al., submitted).

Archaea have been identified in the sponge *Axinella mexicana* (Preston et al., 1996, Schleper et al., 1998). Originally described as *Cenarchaeum symbiosum*, close relatives have since been found in the Australian sponge *Rhopaloeides odorabile*, in Mediterranean sponges of the genus *Axinella*, and in eight different sponge species from the Korean coast (Lee et al., 2003; Margot et al., 2002; Webster et al., 2001a). Interestingly, representatives of this clade have been obtained in pure culture from a seawater aquarium and were shown to be autotrophic and able to oxidize ammonium to nitrite (Könneke et al., 2005). It therefore appears likely that archaeal symbionts use ammonium, which is excreted by sponges as a metabolic end product, as an energy source.

In addition, a novel *Candidatus* phylum 'Poribacteria' has recently been discovered in various marine sponges. The 'Poribacteria' are widely distributed in marine high-microbial-abundance-sponges but have not been found in seawater or sediments. They are characterized by the presence of a nucleoid-containing organelle (Fieseler et al., 2004). Nucleoid-containing bacterial morphotypes were identified in sponges by electron microscopy in early studies (Vacelet and Donadey, 1977), and more recently by Fuerst and coworkers (1999) who also documented the presence of DNA in the nucleoid-like compartments. The morphological diversity of these unusual bacteria encompasses short and long rod-shaped cells as well as D-shaped cells with typical gram-negative cell walls. S-layer structures and bleb-like extrusions of the outer membrane were also noted. Metagenomic library construction and sequencing of a 39 kb Poribacteria-positive fosmid clone showed that the 16S rRNA gene is unlinked from a conventional *rrn* operon (Fieseler et al., 2006). Additionally, a novel kind of molybdenum containing oxidoreductase as well as a series of eight ORFs encoding for unusual transporters and channel or pore forming proteins were identified. This environmental genomics approach provided, for the first time, genomic and, by inference, functional information on the so far uncultivated, sponge-associated candidate division 'Poribacteria'.

V. Horizontal acquisition of symbionts from seawater

Because of the massive filtration rates of sponges, the hypothesis that the microbial symbionts are acquired from seawater was tested. Feeding experiments with six taxonomically different bacterial isolates were performed and the uptake rates were determined over time by plating seawater aliquots to determine the number of colony forming units in the incubation water

(Wehrl et al., in press). Furthermore, feeding experiments were performed with enriched microbial seawater consortia and sponge symbiont consortia that had been obtained by physical separation following established protocols (Fieseler et al., 2006). Because the sponge symbiont consortia and seawater consortia contain a large fraction of uncultivated microorganisms, their uptake rates were determined by DAPI-counting. The uptake rates of the pure cultures and the microbial seawater consortia were very efficient and fell in the same high range (up to 2.76 x 10^6 bacteria g^{-1} sponge wet weight $hour^{-1}$). In contrast, the uptake of sponge symbiont consortia was significantly reduced by almost two orders of magnitude. The internal processing of ingested particles and bacteria in the mesohyl of *A. aerophoba* was documented microscopically. While the GFP-labelled *Vibrio* sp. was immediately digested upon contact with the mesohyl, fluorescent latex beads were taken up efficiently and appeared in the mesohyl in concentric rings. Rhodamine-live-labelled symbionts did not enter the mesohyl at all (Wehrl, 2006). While the reasons for the apparent resistance of the symbionts to uptake and digestion remain unknown, these results provided no evidence for horizontal uptake of symbionts from seawater. However, symbiont acquisition from seawater may still occur in selected instances or by chance and therefore, this hypothesis should not be discarded entirely for the lack of experimental data.

VI. Vertical transmission of symbionts through the reproductive stages

Vertical transmission has been documented in marine bryozoans (Haygood and Davidson, 1997; Lim and Haygood, 2004) and bivalves (Cary and Giovannoni, 1993). Since microorganisms had been visualized in the reproductive stages of various sponges by electron microscopy (Ereskovsky et al., 2005 and references cited therein) the hypothesis was followed whether symbionts are transmitted vertically in the sponge *Ircinia felix* (Schmitt et al., in revision) and several other species (Schmitt et al., submitted) using a combination of electronmicroscopical and molecular techniques.

I. felix is a common, ball-shaped sponge of the Caribbean (Fig. 2A) which produces parenchymella-type larvae in synchronized spawning events. Electronmicroscopical inspection revealed large amounts of morphologically diverse microorganisms in the adult and in the center of larvae that were collected singly from the water column (Fig. 2B). In *I. felix* juveniles, bacteria were found in between densely packed sponge cells. Denaturing

gradient gel electrophoresis (DGGE) revealed highly similar microbial profiles of the adult sponge with that of its reproductive stages. In total, over 200 bands were excised and sequenced. The phylogenetic diversity of adult *I. felix* and its larvae was equally high and included members of the phyla *Alpha-*, *Gamma-* and *Deltaproteobacteria*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Cyanobacteria*, *Bacteroidetes* and a clade of uncertain taxonomic affiliation. 63% of the adult, 54% of the larval and 26% of the juvenile sequences were composed of members of the sponge-specific microbial community described previously (Hentschel et al., 2002).

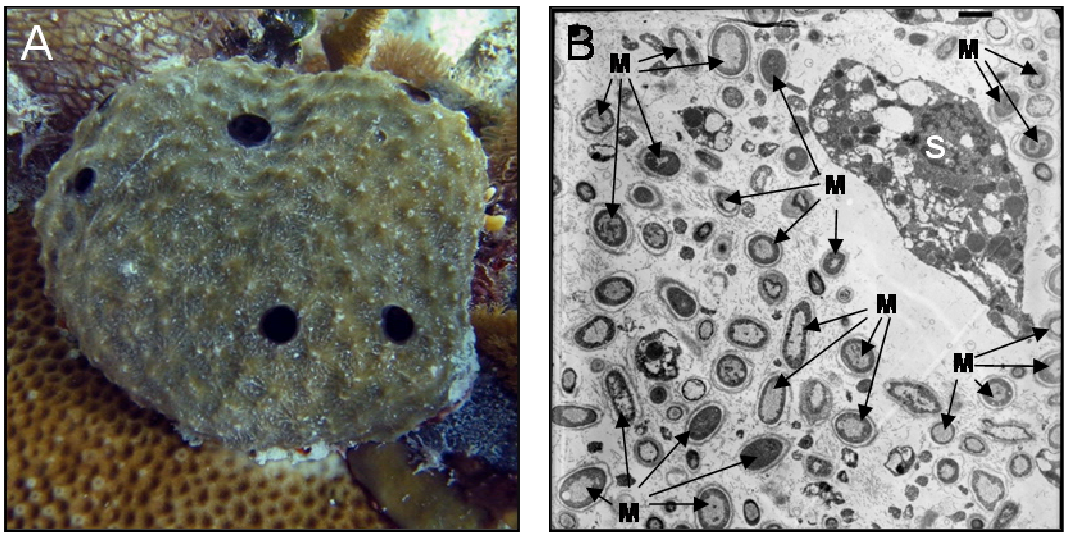

Fig. 2: (A) The bacteriosponge *Ircinia felix* (Irciniidae) from the Florida Keys, USA. (B) Transmission electronmicrograph of *I. felix* larvae collected from the water column. M: Microorganisms, S: Sponge cell. Scale bar: 1 µm. Underwater picture and electron micrograph by S. Schmitt.

In summary, it could be shown that phylogenetically complex microbial consortia are transmitted vertically to the next *I. felix* sponge generation. These results are consistent with Sharp et al. (in press) who reported on the presence of phylogenetically strikingly similar microbial consortia throughout the embryonic development of the tropical sponge, *Corticium* sp.. Moreover, Enticknap et al. (2006) showed vertical transmission of culturable, sponge-specific *Alphaproteobacteria* through the larvae of *Mycale laxissima.* These cumulative results help to gain a better understanding of the evolution and ecology of this possibly very ancient sponge-bacteria association.

VII. Conclusions

In comparison to the many pathogenic and symbiotic types of interactions, the commensal ones have been traditionally the most difficult to study. However, the understanding of commensal microbial consortia is of high importance because bacteria rarely exist as monocultures in nature. It is well known that complex microbial consortia play important roles in various ecological contexts (i.e., human intestine, cattle rumen, marine sponges) and it is becoming increasingly clear that they also have an immediate effect on the nutrition, immune system and the development of their invertebrate and vertebrate hosts (Hooper and Gordon, 2001; Hentschel et al., 2003a). With the implementation of molecular tools for microbial community analysis, it is now possible to define the members of the microbial communities (culturable and non-culturable), and to analyze their functions in the symbiosis context, as has been shown for marine sponges in this review. Studies of this kind will lead to a more comprehensive understanding of commensal bacteria-host interactions that goes well beyond the one-bacterium-one-host concept.

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Chapter Six

Comprehensive phylogenetic analysis reveals the vertical transmission of a uniform yet phylogenetically complex microbial community in six bacteriosponges

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I. Abstract

Many sponge species are permanently associated with large amounts of morphologically and phylogenetically diverse microbial consortia. To elucidate the mechanism by which this association is established and maintained, the microbial community of four Caribbean sponge species (*Agelas wiedenmayeri, Ectyoplasia ferox, Smenospongia aurea, Xestospongia muta*) and their respective reproductive stages was analyzed. Electron microscopy revealed morphologically complex microbial consortia in the four sponge species and their respective reproductive stages. DGGE and 16S rDNA library construction yielded in more than 200 sequences which were affiliated with at least 13 eubacterial phyla. These results demonstrate that highly diverse microbial consortia are present in sponge reproductive stages. Phylogenetic tree construction resulted in 24 vertical transmission clusters (VT-clusters) that, by definition, contain sponge-derived sequences from both, adult and offspring. Therefore, complex, specific, and highly similar microbial communities are vertically transmitted in different sponge species. This is the first comprehensive analysis of the microbiology of sponge reproductive material and it provides experimental support for a highly evolved and possibly ancient microbe-metazoan association.

II. Introduction

Sponges are evolutionarily ancient Metazoa with a simple morphology lacking true organs or tissues (Brusca and Brusca, 1990). Their entire body is adapted to a sessile filter-feeding life style. An aquiferous system consisting of inhalant and exhalant canals is embedded in a massive extracellular matrix, termed the mesohyl. Large volumes of seawater are pumped through the canals and provide the sponge with oxygen and food. Microorganisms and small unicellular eukaryotes are taken up from the seawater with high efficiency leaving the expelled water essentially sterile (Pile 1997, Reiswig 1974, Turon et al. 1997, Wehrl et al. 2007). In addition to these food microorganisms many sponge species of the class Demospongiae are permanently associated with microbes (high-microbial-abundance sponges) (Hentschel et al. 2003, 2006). These microorganisms are located mainly extracellularly in the mesohyl in high concentrations contributing up to 40% of the sponge biomass and thereby exceeding microbial seawater concentrations by two to four orders of magnitude (Friedrich et al. 2001, Webster and Hill 2001).

Already in the 1970s microscopic studies revealed the presence of different microbial morphotypes in sponges based on cell shapes (rods, cocci etc.) and membrane structures (additional membranes, slime layers etc.) (Vacelet 1975, Vacelet and Donadey 1977, Wilkinson 1978). More recent molecular studies using the 16S rDNA as a marker showed the phylogenetic complexity of the microbial community including representatives of at least 9 different eubacterial phyla (Fieseler et al. 2004, Hentschel et al. 2002, Hill et al. 2005, Olson and McCarthy 2005, Taylor et al. 2005, Webster et al. 2001c). Additionally, archaeal representatives of the phylum Crenarchaeota have been detected in several sponge species (Preston et al. 1996, Webster et al. 2001a, Margot et al. 2002, Lee et al. 2003, Holmes and Blanch 2006). Despite the high microbial diversity there seems to be a high degree of uniformity among sponge-associated microbial communities from taxonomically disparate demosponge species. Furthermore, temporal and spatial variability was generally found to be low (Friedrich et al. 2001, Taylor et al. 2004) with the exception of the microbial community in *Cymbastela concentrica* that varied little over 500km along the temperate coast of Australia but was quite different to the one in *C. concentrica* from the tropical coast (Taylor et al. 2005). Stability of the sponge microbiota was shown in perturbation experiments (exposure to antibiotics, starvation conditions, *in situ* transplantation) (Friedrich et al. 2001, Thoms et al. 2003). Only exposure to sub lethal copper concentrations resulted in remarkably decreased microbial numbers and diversity (Webster et al. 2001b).

There are at least two possible ways by which sponges may acquire their microbial associates. First, microorganisms may be taken up horizontally from seawater during filtration. However, feeding experiments with sponge microbial associates that had been obtained by physical separation from sponge tissue showed essentially no uptake in contrast to seawater bacterial isolates that were taken up with high efficiency (Wehrl et al. in press). Alternatively, microorganisms may be vertically transmitted via sponge reproductive stages. Indeed, various microbial morphotypes have been detected by electron microscopy in oocytes, embryos and larvae of viviparous as well as oviparous sponge species (Ereskovsky et al. 2005 and references cited therein). The only culture-based study to date succeeded in isolating an alphaproteobacterial strain from adult and embryo samples of the sponge *Mycale laxissima* (Enticknap et al. 2006). The same strain had previously been detected in several other sponge species (Scheuermayer et al. 2006). Two recent molecular studies found a phylogenetically complex microbial community in embryos and larvae of the sponge species *Corticium* sp. and

Ircinia felix, respectively and vertical transmission of several phylotypes through different reproductive stages was shown (Schmitt et al. 2007, Sharp et al. 2007).

For this study the oviparous sponges *Agelas wiedenmayeri*, *Ectyoplasia ferox,* and *Xestospongia muta* as well as the viviparous species *Smenospongia aurea* from the Caribbean were chosen. The first three species release oocytes that are embedded in a mucous sheath whereas *S. aurea* broods its embryos and releases free swimming larvae into the water column. Adult and reproductive samples were investigated with a combination of microscopic (TEM) and molecular methods (DGGE, 16S rDNA library) to compare the microbial profiles of different developmental stages. The aim of this study was to provide a better understanding of the importance of vertical transmission for the establishment and maintenance of the association of marine sponges with complex microbial consortia.

Table 1: Compilation of sponges of which adult and reproductive material derived 16S rDNA sequences are available.

Host sponge (order[1])	Sequence source	Collection site (latitude / longitude)	GenBank accession no.	Reference
Agelas wiedenmayeri (Agelasida)	DGGE[3]	Key Largo, FL (25°02′N / 80°24′W)	EF159732-39	this study
Corticium candelabrum (Homosclerophorida)	16S rDNA library	Palau islands (07°23′ N / 134°38′E)	DQ247938-52, DQ247954, DQ247956-57	Sharp et al. (2007)
Ectyoplasia ferox (Poecilosclerida)	DGGE[3]	Key Largo, FL (25°02′N / 80°24′W); Bahamas islands (24°32′N / 75°55′W)	EF159740-72	this study
Ircinia felix (Dictyoceratida)	DGGE[3]	Key Largo, FL (25°02′N / 80°24′W)	DQ661746-857	Schmitt et al. (2007)
Mycale laxissima (Poecilosclerida)	Isolates	Key Largo, FL (25°02′N / 80°24′W)	DQ097238, DQ097259	Enticknap et al. (2006)
Smenospongia aurea (Verongida[2])	DGGE[3]	Key Largo, FL (25°02′N / 80°24′W)	EF159773-833	this study
Xestospongia muta (Haplosclerida)	16S rDNA library	Key Largo, FL (25°02′N / 80°24′W)	EF159834-941	this study

[1] classified after "Systema Porifera" by Hooper and van Soest (2002)
[2] reclassified after Schmitt et al. (2005)
[3] sequencing of excised DGGE bands

III. Material and Methods

Sponge collection

The sponges *Agelas wiedenmayeri, Ectyoplasia ferox, Smenospongia aurea* and *Xestospongia muta* were collected by SCUBA diving offshore Key Largo, Florida in June 2002, June and August 2004 and in May 2005 (Table 1). Additional material of *E. ferox* was collected by SCUBA diving offshore French Wells, Bahamas in July 2003. Reproductive material (oocytes, larvae) and adult samples of the corresponding sponge individuals were collected using the methodology of Lindquist et al. (1997). After the adult samples were cut into small pieces, the adult and reproductive material was washed in sterile filtered seawater, fixed either in 96% ethanol or in 2.5% glutaraldehyde/H_2O_{dd} and stored at -20°C. *In-vivo*-settlement experiments were performed with a portion of the captured larvae of *S. aurea* as described by Schmitt et al. (2007). Briefly, larvae were placed in sealed plastic containers (~75 ml vol) for settlement and juveniles were recovered 1-3 day post-settlement and treated as described above.

Transmission electron microscopy (TEM)

Adult samples that had been fixed in 2.5% glutaraldehyde/$H_2O_{dd,}$ were cut into small cubes of about 1mm^3. All samples (adult, larvae, juveniles) were then washed five times in cacodylate buffer (50 mM, pH 7.2), fixed in 2% osmium tetroxide for 90 min, washed again five times in H_2O_{dd} and incubated overnight in 0.5% uranyl acetate. After dehydration in an ethanol series (30, 50, 70, 90, 96, 3x100% for 30 min each), samples were incubated 3x30 min in 1x propylene oxide, overnight in 1:1 (v/v) propylene oxide/Epon 812 (Serva), 2x2 h in Epon 812 and finally embedded in Epon 812 for 48 hours at 60°C. Samples were then sectioned with an ultramicrotom (OM U3 C. Reichert, Austria) and examined by transmission electron microscopy (Zeiss EM 10, Zeiss, Germany).

Extraction of DNA and 16S rDNA library construction

The adult samples that had been fixed in 96% ethanol were air-dried, ground with a mortar and pestle and DNA was extracted using the Fast DNA Spin kit for soil (Q-Biogene, Heidelberg, Germany) in accordance with the manufacturer`s instructions. 3, 5 or 7 individual larvae or juveniles, which had been pooled immediately after collection, were also air-dried, transferred into 150µl H_2O_{dd} and DNA was extracted by heating the samples in a water bath for 10 min at 100°C. The solution was used directly as template in the following PCR.

The universal primers 27f and 1492r (Lane 1991) were used for PCR amplification of eubacterial 16S rDNA. Cycling conditions were as follows: initial denaturing step at 96°C for 5 min, 30 cycles of denaturing at 96°C for 1 min, primer annealing at 54°C for 1 min and elongation at 72°C for 1.5 min, followed by a final extension step at 72°C for 5 min.

Denaturing Gradient Gel Electrophoresis (DGGE)

DNA was extracted as described above. The universal primers 341f with GC-clamp and 907r (Muyzer et al. 1998) were used for PCR amplification of eubacterial 16S rDNA. Cycling conditions on a Mastercycler Gradient (Eppendorf, Hamburg, Germany) were as follows: initial denaturing step at 95°C for 5 min, 30 cycles of denaturing at 95°C for 1 min, primer annealing at 54°C for 1 min and elongation at 72°C for 45 sec, followed by a final extension step at 72°C for 10 min. DGGE was performed with a Bio-Rad DCode Universal Mutation Detection System (Bio-Rad, München, Germany) on a 10% (w/v) polyacrylamide gel in 1x TAE and using a 0–90% denaturing gradient; 100% denaturant corresponded to 7 M urea and 40% (v/v) formamide. Electrophoresis was performed for 6 h at 150 V and 60°C. Gels were stained for 30 min in SYBR Gold (Molecular Probes) and scanned on a Typhoon 8600 scanner (Amersham Biosciences). Selected bands were excised with an ethanol sterilized scalpel and incubated in 25µl H_2O_{dd} overnight at 4°C. 4 µl of eluted DNA was subsequently used for reamplification with primers 341f and 907r under PCR conditions described above.

Cloning, sequencing and phylogenetic analysis

PCR products of clones from excised DGGE bands and of the 16S library were ligated into the pGEM-T-easy vector (Promega) and transformed by electroporation into competent *E. coli* XL 1-Blue cells. Plasmid DNA was isolated by standard miniprep procedures and the correct insert size was verified using agarose gel electrophoresis following restriction digestion. Additionally, the clones from the 16S library were characterized by single digestion with the restriction endonucleases HaeIII and Sau3A I (restriction fragment length polymorphism (RFLP) analysis). Clones with identical restriction patterns were grouped together, and random clones from each group were chosen for sequence analysis. Sequencing was performed on an ABI 377XL automated sequencer (Applied Biosystems) and sequences were edited with the ContigExpress Tool in Vector NTI suite 6.0 (InforMax, Inc). Sequences were checked for chimeras with the program Pintail (Ashelford et al. 2006) and for other amplification and sequencing artifacts. Following removal of chimeras from the dataset, percentage similarities (p-distances) between sequences from the same source (adult, larvae,

juvenile) were determined with the editor Align (Hepperle 2002) and those with identities above 99% were grouped together into operational taxonomic units (OTUs). Only one randomly chosen sequence per OTU was used for further analysis. Sequences obtained in this study together with reference sequences (all nearest BLAST matches and, moreover, representatives of the respective phylum) were aligned automatically with ClustalX (Thompson et al. 1997) and the alignment was subsequently corrected manually in Align (Hepperle 2004). Phylogenetic trees were constructed with the ARB software package (Ludwig et al. 2004). Initially, neighbour-joining (Jukes-Cantor correction) trees were calculated with nearly full length sequences (>1250bp) and 100 pseudoreplicates. Subsequently, partial sequences were added to the tree without changing the topology by the use of the parsimony-interactive method in ARB.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences were deposited in EMBL/GenBank/DDBJ under accession numbers EF159732-EF159941.

IV. Results

Transmission electron microscopy

The mesohyl of adult *A. wiedenmayeri*, *E. ferox*, and *S. aurea* consisted of an extracellular matrix in which sponge cells and many microorganisms were embedded (Fig. 1A, C, E). Electron microscopy with *X. muta* samples was not performed. The amoeboid-like sponge cells were loosely scattered, 5-8µm in length and phagocytotic active as indicated by the presence of microorganisms in the process of digestion inside the cells (Fig. 1E). Choanocyte chambers were built by specialized, flagellated sponge cells (choanocytes) which were arranged in a circle around the water canal. Four flagella and many microvilli were visible in the cross-section (Fig. 1C). Sponge cells were surrounded by large amounts of morphologically diverse microorganisms. These microorganisms were up to 2.5µm in length, showed different shapes like short or long rods, cocci, D-shaped forms or star-like shapes and varied in membrane structures. Bacterial cell division could also be observed (Fig. 1A, E).

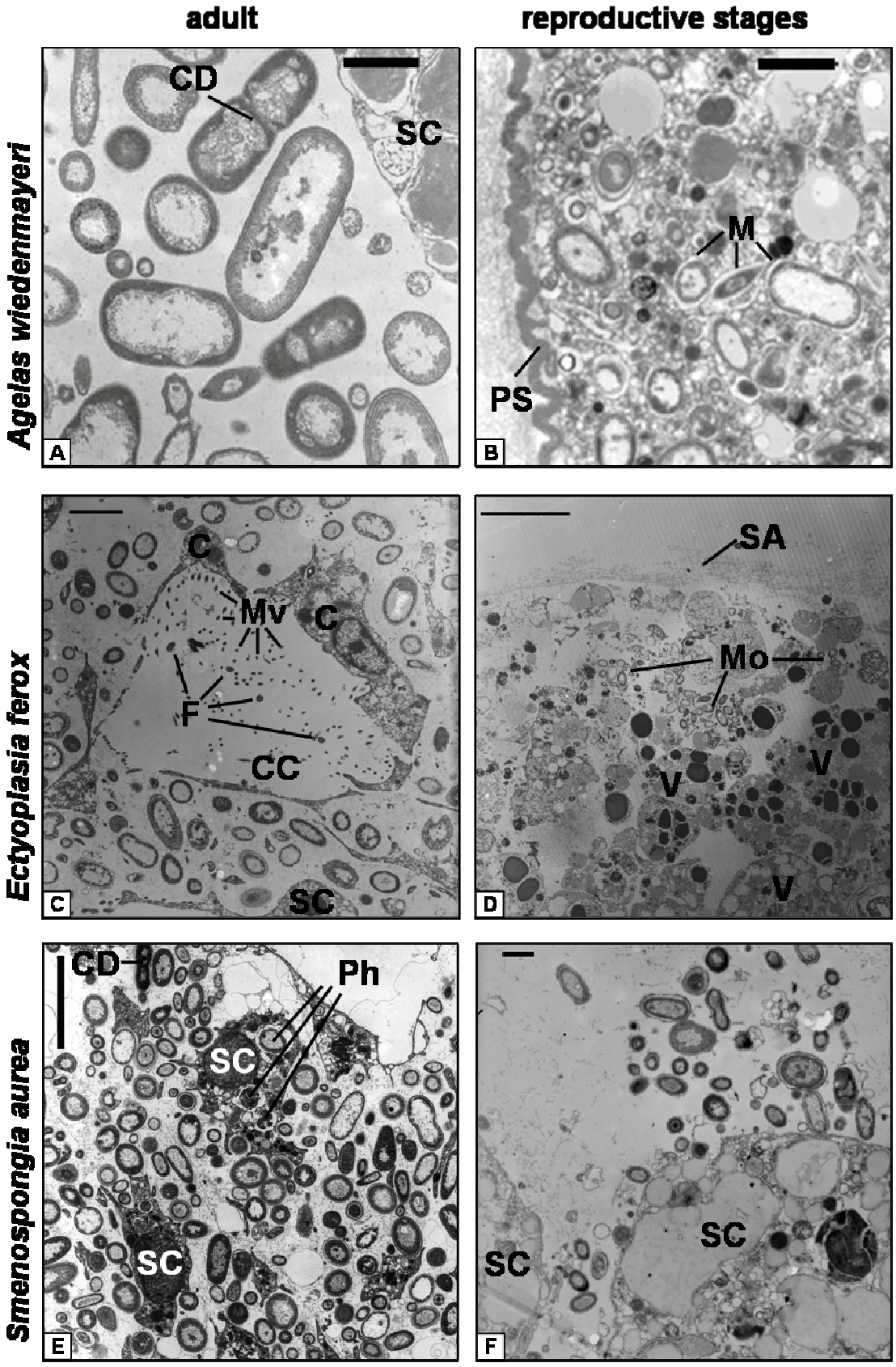

Fig. 1: Transmission electron microscopy of microorganisms present in the sponges *A. wiedenmayeri* adult (A) and embryo (B), *E. ferox* adult (C) and early embryo (D) and *S. aurea* adult (E) and larvae (F). C: choanocytes, CC: choanocyte chamber, CD: bacterial cell division, F: flagella, M: membrane, Mi: microvilli, Mo: microorganisms, Ph: phagocytosis, PS: protein-like structure, SA: surface area, SC: sponge cell, V: vesicle. Scale bar: 1µm (A, B, F), 2µm (C), 5µm (E), 10µm (D).

A. wiedenmayeri and *E. ferox* both release oocytes or early embryonic stages embedded in a matrix. These developmental stages were not ciliated, contained many electron-transparent and also electron-dense vesicles and in the case of *A. wiedenmayeri*, were surrounded by a protein-like structure (Fig. 1B, D). Diverse microorganisms were located inside the reproductive stages of both species. They were similar in sizes, shapes and membrane structures to the microorganisms found in the respective adult sponges. While each microorganism was enclosed by a membrane in *A. wiedenmayeri* (Fig. 1B) no such membranes were detected around microorganisms in *E. ferox* (Fig. 1D). The viviparous species *S. aurea* released free swimming larvae into the water column. Sponge cells in the center of the larvae were filled with vesicles and lipids (Fig. 1F). Microorganisms were extracellular but located closely to the sponge cells. They appeared morphologically similar to the microorganisms in the adult samples. The microorganisms in the reproductive stages of all three species showed no signs of digestion (Fig. 1B, D, F).

Sequence analysis

DGGE bands were excised and DNA reamplified and sequenced. 6 sequences were obtained from *A. wiedenmayeri* (embryo), 31 from *E. ferox* (adult: 6; oocytes: 25) and 61 from *S. aurea* (adult: 39; offspring: 19). For the *X. muta* adult and reproductive material one 16S rDNA library each consisting of 205 clones was constructed. RFLP analysis yielded 47 and 98 unique restriction patterns for the adult and reproductive material library, respectively. After sequencing of one randomly chosen clone per restriction pattern and grouping the sequences into OTUs based on a 99% cut-off, in total 103 sequences were derived (adult: 31; offspring: 72). Figs. 2-6 illustrate the phylogenetic position of sequences within 13 eubacterial phyla.

Vertical transmission clusters (VT-clusters)

Vertical transmission clusters were recently defined as monophyletic clusters of two or more sequences that were recovered from both, the adult sponge and offspring (Schmitt et al. 2007). In this study, 23 VT-clusters were found that were distributed in 8 different eubacterial phyla (Figs. 2-6). One additional VT-cluster could not be affiliated with any known eubacterial phylum (Fig. 4) and is therefore termed Uncertain 1. 92% (18 out of 24) of all VT-clusters contain at least one additional sponge derived sequences. 25% (6 out of 24) of all VT-clusters are double or triple VT-clusters because they contain adult and reproductive stage derived sequences from two or three species.

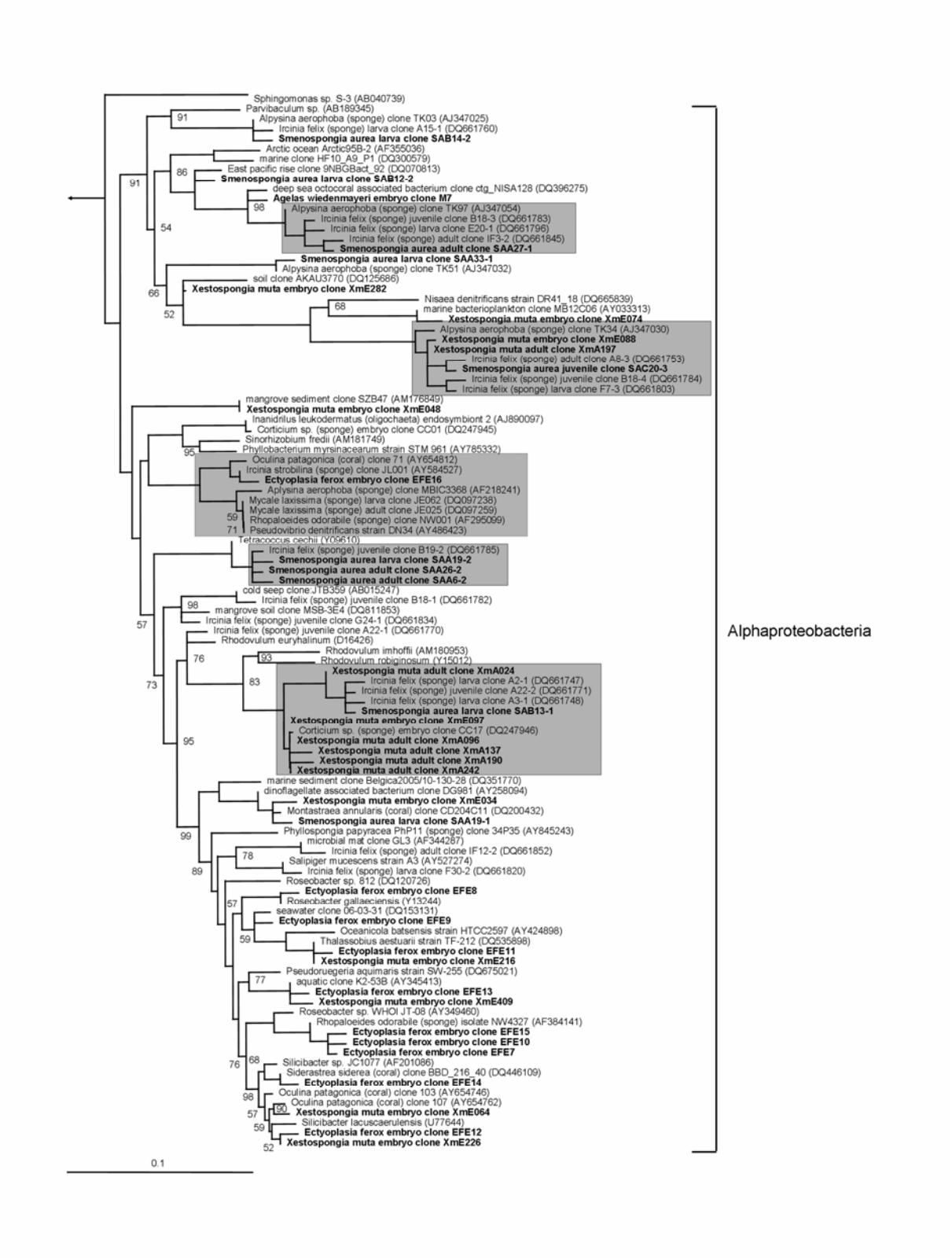

Fig. 2: Phylogenetic distance tree calculated with 16S rDNA partial sequences affiliated with the Alphaproteobacteria. Neighbor-joining (100 pseudoreplicates) bootstrap values are provided. Sequences obtained in this study are given in bold. Boxes depict VT-clusters. The dotted lined box indicates the cluster described by Enticknap et al. (2006). Scale bar indicates 10% divergence. Arrow, to outgroup (*Geothrix fermentans* U41563, *Holophaga foetidae* X77215, *Acidobacterium capsulatum* D26171).

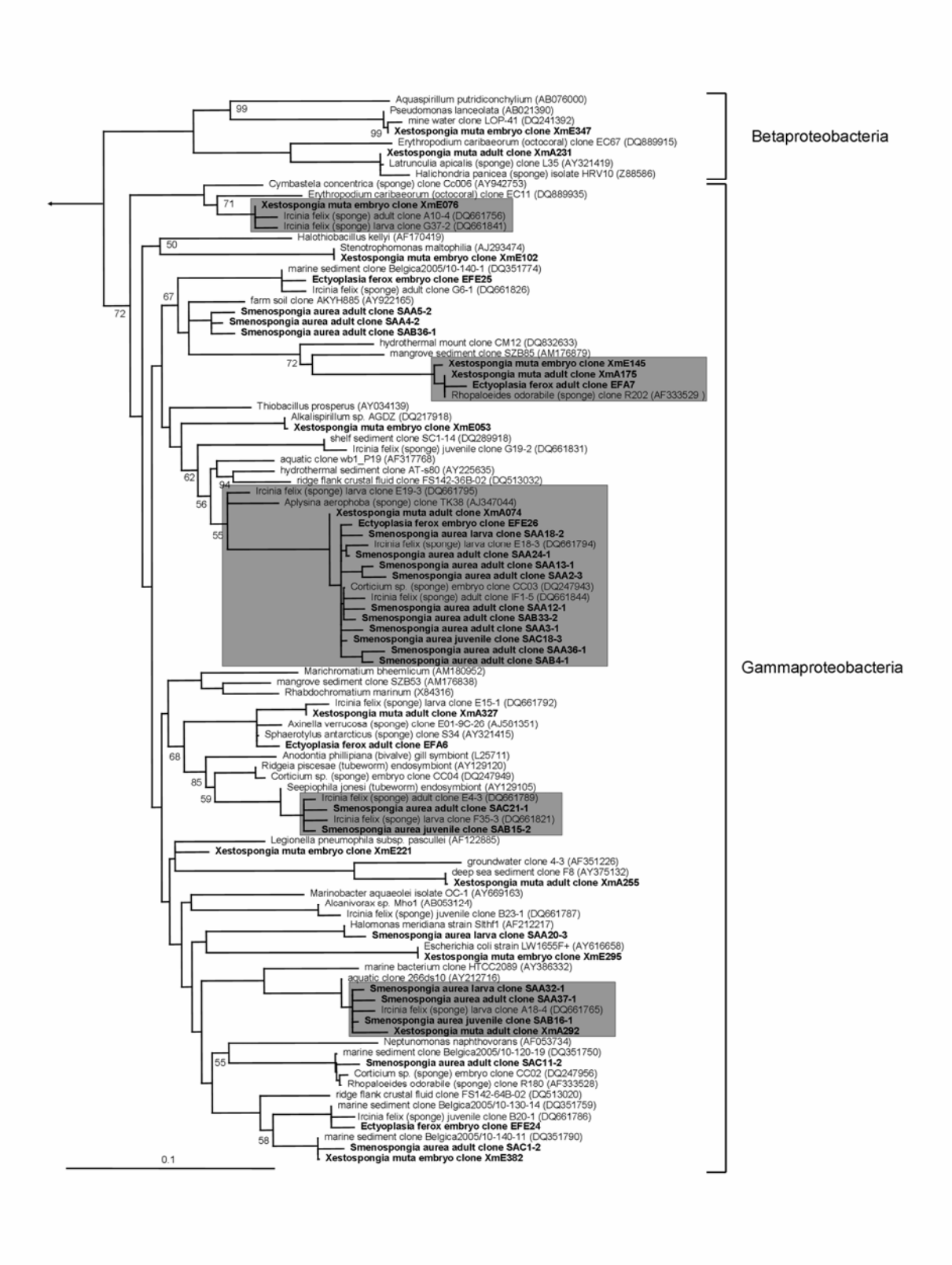

Fig. 3: Phylogenetic distance tree calculated with 16S rDNA partial sequences affiliated with the Beta- and Gammaproteobacteria. Neighbor-joining (100 pseudoreplicates) bootstrap values are provided. Sequences obtained in this study are given in bold. Boxes depict VT-clusters. Scale bar indicates 10% divergence. Arrow, to outgroup (*Geothrix fermentans* U41563, *Holophaga foetidae* X77215, *Acidobacterium capsulatum* D26171).

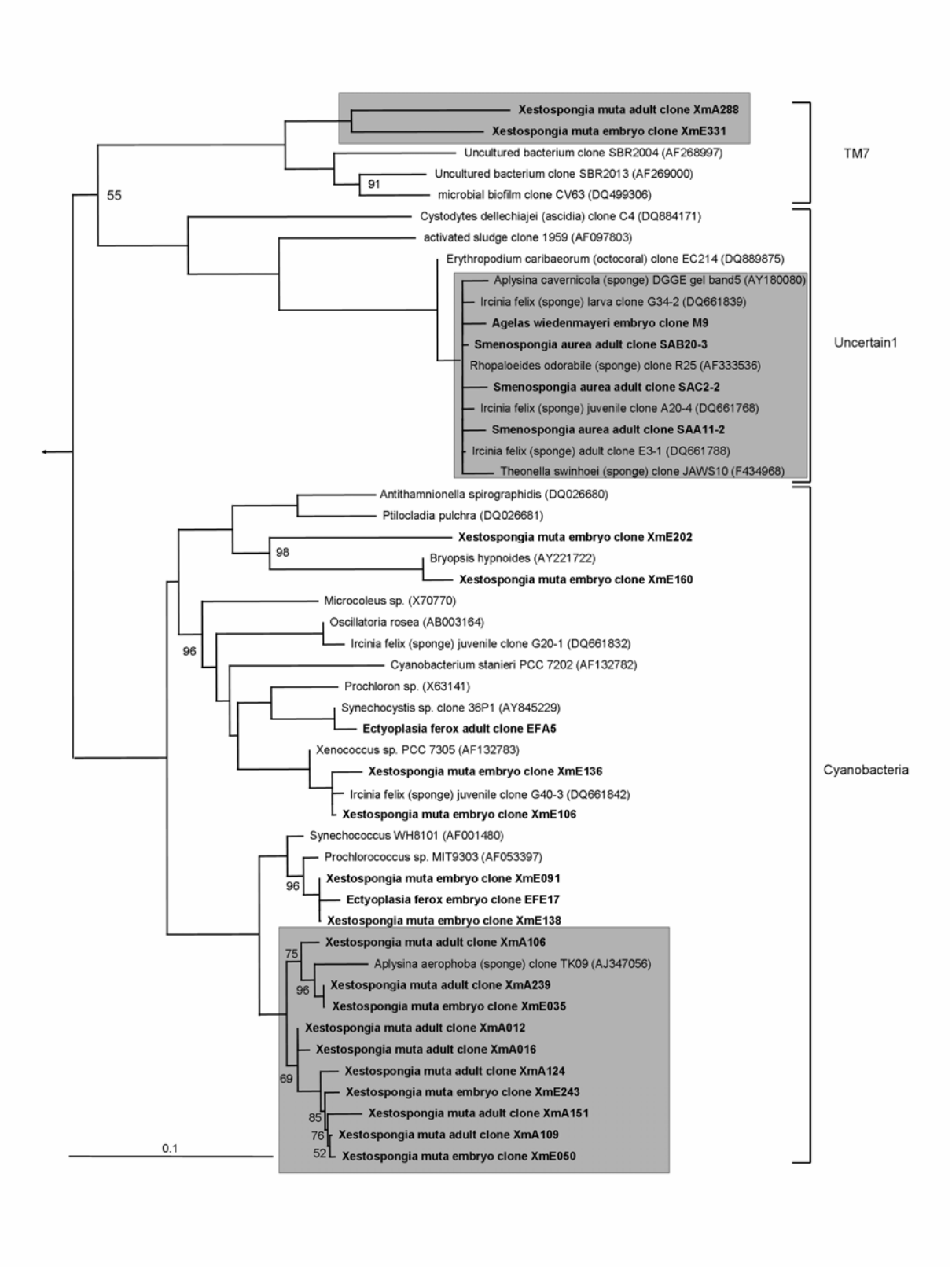

Fig. 4: Phylogenetic distance tree calculated with 16S rDNA partial sequences affiliated with the Cyanobacteria, the TM7 candidate phylum and sequences of uncertain affiliation. Neighbor-joining (100 pseudoreplicates) bootstrap values are provided. Sequences obtained in this study are given in bold. Boxes depict VT-clusters. Scale bar indicates 10% divergence. Arrow, to outgroup (*Geothrix fermentans* U41563, *Holophaga foetidae* X77215, *Acidobacterium capsulatum* D26171).

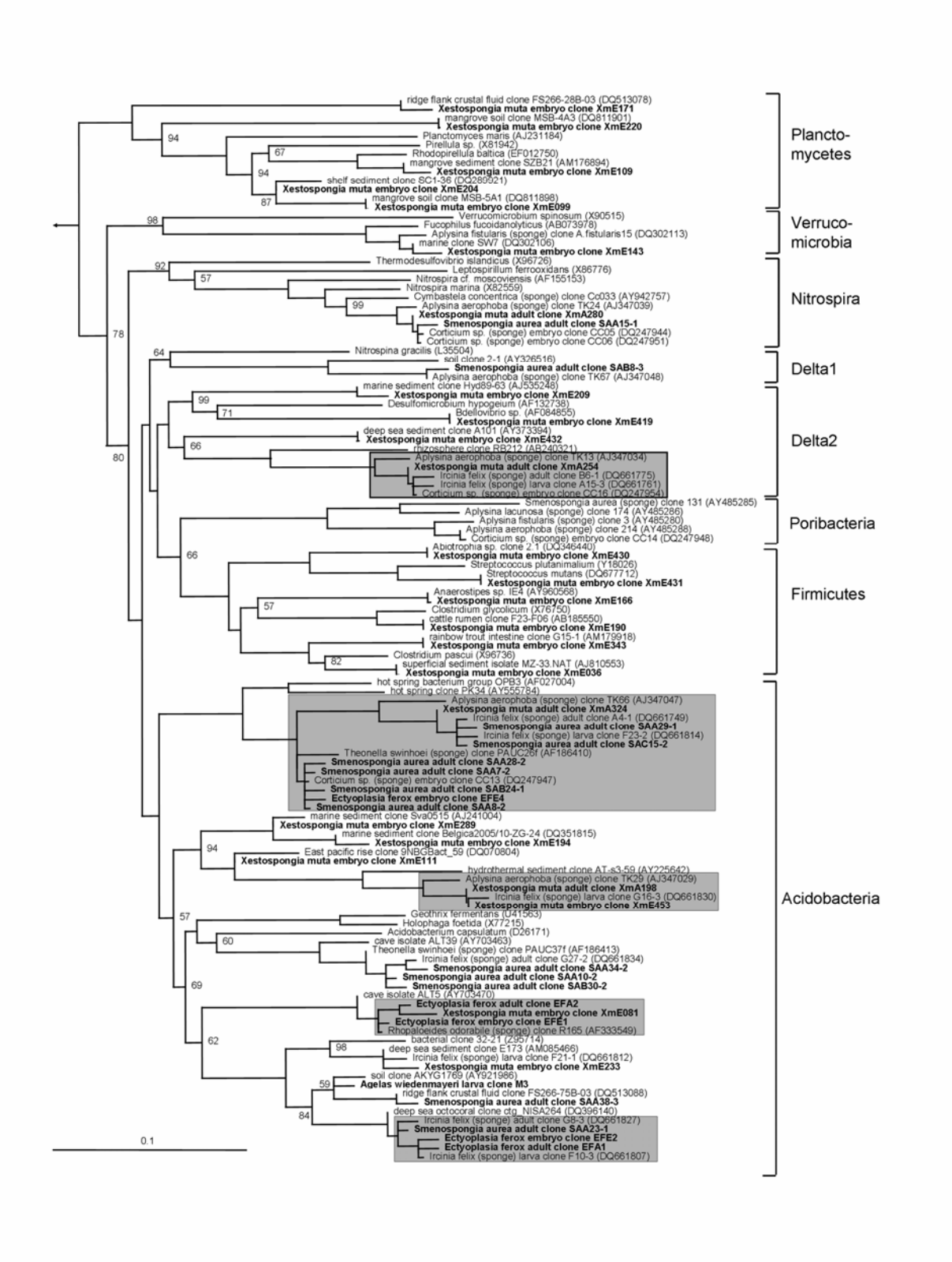

Fig. 5: Phylogenetic distance tree calculated with 16S rDNA partial sequences affiliated with the Planctomycetes, Verrucomicrobia, Nitrospira, Deltaproteobacteria, Poribacteria, Firmicutes and the Acidobacteria. Neighbor-joining (100 pseudoreplicates) bootstrap values are provided. Sequences obtained in this study are given in bold. Boxes depict VT-clusters. Scale bar indicates 10% divergence. Arrow, to outgroup (*Geothrix fermentans* U41563, *Holophaga foetidae* X77215, *Acidobacterium capsulatum* D26171).

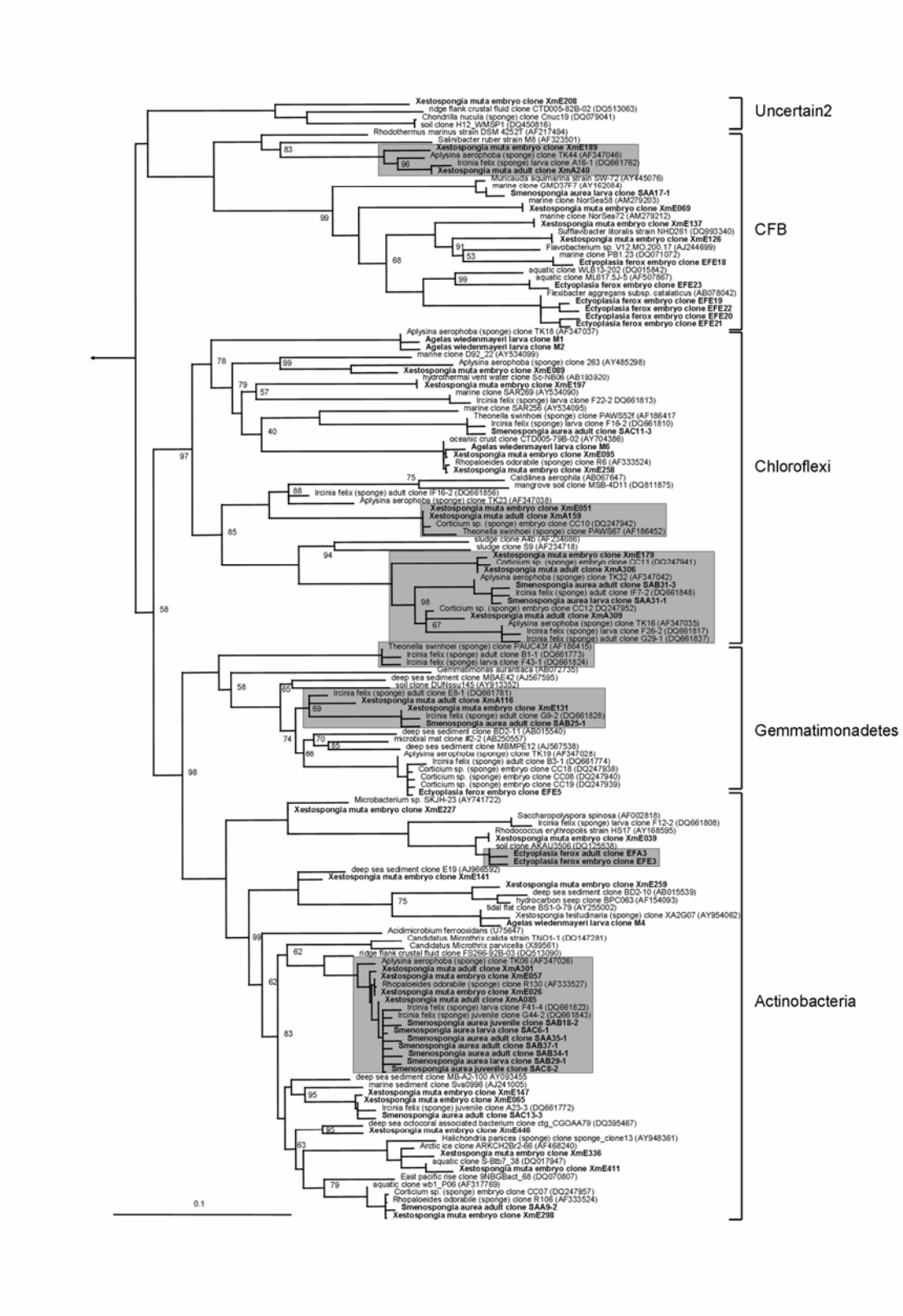

Fig. 6: Phylogenetic distance tree calculated with 16S rDNA partial sequences affiliated with the Bacteroidetes, Chloroflexi, Gemmatimonadetes, Actinobacteria and a sequence of uncertain affiliation. Neighbor-joining (100 pseudoreplicates) bootstrap values are provided. Sequences obtained in this study are given in bold. Boxes depict VT-clusters. Scale bar indicates 10% divergence. Arrow, to outgroup (*Geothrix fermentans* U41563, *Holophaga foetidae* X77215, *Acidobacterium capsulatum* D26171).

V. Discussion

Vertical transmission generally points to a highly adapted and ancient association of microorganisms with their hosts. This mechanism of transferring microorganisms between two host generations has been described for several examples of invertebrates from the terrestrial (e.g. Moran and Baumann 2000, Schröder et al. 1996) and marine environment (e.g. Cary and Giovannoni, 1993, Giere et al. 1991, Haygood and Davidson 1997, Krueger et al. 1996, Sipe et al. 2000). Most of these systems consist of a well defined one host – one (or few) symbiont relationship and vertical transmission is a highly specialized process. In contrast, in the sponge-microbe association different, distantly related sponge species contain a highly similar microbial community that consists of representatives of at least 9 different phyla (Hentschel et al. 2006). Electron microscopy studies first documented microorganisms in sponge reproductive stages and vertical transmission as a mechanism to maintain sponge-microbe associations was proposed already in 1962 by Levi and Porte. The electron micrographs of adult *A. wiedenmayeri*, *E. ferox* and *S. aurea* each revealed morphologically diverse microbial communities in the mesohyl (Fig. 1A, C, E). Similar microbial morphotypes were also present in the respective reproductive stages (Fig. 1B, D, F). Moreover, these morphotypes were previously described from other high-microbial abundance sponges (Friedrich et al. 1999, Vacelet 1975, Wilkinson 1978). The electron micrographs of this study give a first indication that the Caribbean sponges *A. wiedenmayeri*, *E. ferox*, and *S. aurea* also belong to the group of high-microbial abundance sponges. Furthermore, the data suggest that the complex microbial community is vertically transmitted. These data add to a growing body of literature that visually document microorganisms in sponge reproductive stages (Ereskovsky and Tokina 2004, Gallissian and Vacelet 1976, Kaye 1991).

Adult samples of *E. ferox*, *S. aurea*, and *X. muta* contained phylotypes of at least five different eubacterial phyla each. No sequences were obtained from adult *A. wiedenmayeri*. Many sequences of this study cluster with sequences from other high-microbial abundance sponges (Figs. 2-6). From the molecular as well as the microscopic results it can therefore be concluded that the four species of this study harbour the sponge specific microbial community reported previously (Hentschel et al. 2002). One of the *X. muta* derived sequences was affiliated with the candidate phylum TM7 (Fig. 5). Representatives of this phylum are widespread in terrestrial as well as aquatic habitats (Hugenholtz et al. 2001) but have not been detected in other sponges so far. However, the same phylotype was also found in *X. muta*

reproductive stages and might therefore represent a bacterium that is specifically associated and vertically transmitted in *X. muta*.

More than 120 sequences were obtained from reproductive stages of *A. wiedenmayeri, E. ferox, S. aurea* and *X. muta* in this study and analyzed with over 55 published sequences of other sponge reproductive stages (Enticknap et al. 2006, Schmitt et al. 2007, Sharp et al. 2007). From this comprehensive data set, a total of 24 vertical transmission clusters were found in 8 different eubacterial phyla. The phyla Firmicutes, Planctomycetes and Verrucomicrobia only contain *X. muta* reproductive material derived sequences and these sequences are not closely related to sequences from other sponges (Fig. 5). It might be possible that these sequences represent seawater microorganisms that were growing in the sheath in which the reproductive material is embedded although it cannot be completely excluded that the same phylotypes are also present in adult *X. muta* but were not detected with the methods used. Nitrospira affiliated sequences were obtained from adult *S. aurea* and *X. muta* but not from the respective reproductive material (Fig. 5). However, these sequences cluster together with other sponge derived sequences among them two from embryos of the sponge *Corticium* sp. It seems therefore most likely, that this phylotype is also vertically transmitted. Poribacteria were not detected in this study because of differences in the primer region but a *Corticium* sp. embryo derived sequence clusters among other poribacterial sequences again indicating vertical transmission. Currently a study with specific primers is ongoing to test for the presence of vertical transmission clusters in these two phyla. The alphaproteobacterial strain that has been detected in *M. laxissima* adult and embryos is >99% identical to the environmental sequence of *Pseudovibrio denitrificans* (AY486423) and does not match the definition of a VT-cluster. Although closely related strains were isolated from different sponges not all of these sponges contain large amounts of microorganisms in their mesohyl as shown by transmission electron microscopy and are therefore not regarded as high-microbial abundance sponges (Schmitt et al. submitted). It is possible, that this alphaproteobacterial strain represents another, more general type of association with sponges.

25% of all clusters are double VT-cluster because they contain adult and reproductive material derived sequences of two species. All double VT-clusters contain additional sponge derived sequences except of one. Noteworthy is one cluster within the phylum Chloroflexi because it contains adult and reproductive material derived sequences of the three species *I. felix*, *S. aurea* and *X. muta*. Moreover, *Corticium* sp. embryo sequences and sequences from

the Mediterranean sponge *Aplysina aerophoba* are closely affiliated whereas the next environmental sequences (sludge clones S9 and A4b; AF234718, AF234686) show less than 86% similarity. Apparently, the same phylotypes are vertically transmitted in geographically and phylogenetically distinct sponge species.

The studies of Schmitt et al. (2007) and Sharp et al. (2007) both documented that phylogenetically complex microbial consortia are vertically transmitted in each of the sponges *I. felix* and *Corticium* sp.. In this study the combined and expanded data set reveals that vertical transmission is a widespread phenomenon in sponges. Moreover, vertical transmission appears to be a highly selective process by which exclusively the members of the sponge specific microbial community are passed on to the next generation. At the same time, vertical transmission is widespread among the high microbial abundance sponges and seems not to be affected by the sponge phylogeny, mode of reproduction or geography as *A. wiedenmayeri*, *E. ferox*, *X. muta*, and *S. aurea* as well as *I. felix* and *Corticium* sp. all belong to different orders, represent two different modes of reproduction (the former three are oviparous, the latter three are viviparous) and grow in different geographical areas (*Corticium* sp. from Palau, remaining species from the Caribbean).

In summary, the microscopic and molecular data revealed that highly similar microbial consortia are vertically transmitted in the sponges *A. wiedenmayeri*, *E. ferox*, *X. muta*, and *S. aurea*. For the first time all sponge reproductive material derived sequences were combined in a comprehensive analysis. The results confirm and expand recent results (Schmitt et al. 2007, Sharp et al. 2007) and allow generalizing the process of selective transmission of sponge specific microbes in high-microbial abundance sponges. Vertical transmission is therefore an important mechanism by which the probably long existing association of demosponges and complex microbial communities is maintained.

VI. Acknowledgements

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Chapter Seven
General Discussion

Often one organism influences the evolution of another organism, but not vice versa; this has been termed sequential evolution. In a specific and close relationship the interaction might have a reciprocal influence and the partners coevolve over time resulting in reciprocal adaptations or coadaptations (for reviews see Futuyama and Slatkin 1983, Ridley 1996, Thompson 1994). The reconstruction of genealogical relationships among taxa and the comparison of phylogenies of hosts and associated partners allow the study of the evolutionary history and the origin of interactions. If the phylogenies mirror each other, the speciation events in both partners occurred simultaneously. Cospeciation depends to a certain degree on the dispersal ability of the associated partner. If one partner lives strictly associated with another one and is not found independently it will automatically cospeciate with its partner. In most cases the intimate association of cospeciated partners is maintained by strict vertical transmission of one partner via reproductive stages of the other one. On the contrary, intimate and obligate associations do not have to have evolved in cospeciation and can be maintained in addition to vertical transmission by horizontal uptake of the associated partner from another host individual or from the environment.

Among the invertebrate phyla many species live in intimate associations with microorganisms whereby the microbes can be located extracellularly or intracellulary. Often these associations are mutualistic and obligate and are maintained either by vertical transmission or by horizontal acquisition. In the terrestrial environment diverse insect groups such as aphids, carpenter ants, and cockroaches harbour primary symbionts in specialized cells (bacteriocytes) and often additional secondary symbionts that are not always confined to bacteriocytes (Moran and Baumann 2000). Both types of symbionts are vertically transmitted although the secondary symbiont might also be transferred horizontally between hosts (Bandi et al. 1995, Clark et al. 2001a, Sandström et al. 2001, Schröder et al. 1996). Accordingly, cospeciation was proposed for the primary but not for the secondary symbiont. In the marine environment chemoautotrophic bacteria are associated with a wide range of invertebrate hosts that belong to at least five animal phyla including Mollusca, Pogonophora, and Vestimentifera (Cavanaugh 1994, Fisher 1990). Although the microbial functions are similar (oxidation of

reduced sulphur compounds, fixation of inorganic carbon), the associations are maintained in some cases by environmental acquisition (e.g. lucinid clams, vestimentiferan tube worms) (Feldman et al. 1997, Gros et al. 1996) and in others by vertical transmission (e.g. solemyid bivalves, vesicomyid clams) (Cary and Giovannoni 1993, Hurtado et al. 2003, Krueger et al. 1996) that might lead to cospeciation (Peek et al. 1998). The deep-sea *Bathymodiolus* mussels are special in that they have an environmental "leaky" system of vertical transmission (Won et al. 2003). A remarkable example of cospeciation without vertical transmission of the symbionts is the sepiolid squid – *Vibrio fischeri* symbiosis (Nishiguchi et al. 1998). These partners show parallel evolution yet the microorganisms are newly acquired horizontally by reinfecting each generation.

I. Cospeciation of *Aplysina* sponges and cyanobacteria

Aplysina phylogeny

The genus *Aplysina* (order Verongida) was chosen for reconstruction of host phylogeny. *Aplysina* sponges are well known to contain large and complex microbial consortia and the Mediterranean species *A. aerophoba* is one of the most intensively studied systems in this regard (Fieseler et al. 2004, Friedrich et al. 2001, Hentschel et al. 2001, 2002, Vacelet 1975). However, the assignment of species to *Aplysina* and the relationships within this genus are still uncertain because several taxonomic features that were thought to be characteristic for *Aplysina* are now recognized to be shared with other verongid groups (Hooper and van Soest 2002).

In this Ph.D. thesis two Mediterranean and six Caribbean *Aplysina* species were analyzed (chapter 2). The genus *Aplysina* appeared as monophyletic in the 18S rDNA phylogeny but was supported up to only 75%. Within the *Aplysina* cluster no or only weak resolution (e.g. low bootstrap support values) was obtained. In the ITS-2 phylogenetic tree, a soft form of *A. lacunosa* stands at the basis followed by a Mediterranean cluster including *A. aerophoba* and *A. cavernicola* and by a second cluster that contains all the remaining Caribbean and Eastern Pacific *Aplysina* species. The basal position of *A. lacunosa* soft is in agreement with the 18S rRNA phylogeny and is additionally confirmed by cytochrome oxidase subunit 1 (COI) sequence comparison (Table 1). Moreover, subsequent morphological inspection revealed a more open and irregular fibre skeleton than the typical *Aplysina* spongin skeleton. All these

data suggest an assignment of *A. lacunosa* soft to another genus of the order Verongida. Within the Caribbean – Eastern Pacific cluster of the ITS-2 phylogeny no further resolution was obtained due to highly similar sequences. Similarly, also COI sequences of *Aplysina* sponges are highly conserved (Table 1). The clustering of *A. aerophoba* and *A. cavernicola* against all other *Aplysina* species resembles the geographic distribution of these sponges. Based on the ITS-2 phylogeny the following biogeographic scenario would be conceivable: a possibly Tethyan ancestor for all *Aplysina* resulted in a Mediterranean clade and in a much more diverse Caribbean – Eastern Pacific clade. Assuming that the process of adaptive radiation that resulted in all extant Caribbean – Eastern Pacific *Aplysina* occurred only recently, then this would explain the high degree of similarity between these genes.

Table 1: COI sequence similarities between *Aplysina* sponges

	A. aerophoba	*A. cavernicola*	*A. archeri*	*A. fulva*	*A. cauliformis* thin	*A. lacunosa* hard	*A. lacunosa* soft
A. aerophoba	-	99.6%	99.7%	99.3%	99.4%	99.4%	91.7%
A. cavernicola		-	99.6%	99.2%	99.3%	99.3%	91.4%
A. archeri			-	99.7%	99.6%	99.7%	91.8%
A. fulva				-	99.4%	99.2%	91.4%
A. cauliformis thin					-	99.4%	92.0%
A. lacunosa hard						-	92.9%
A. lacunosa soft							-

Comparison of sponge and cyanobacterial phylogeny

Various studies have focused on the identification of cyanobacteria in sponges (Steindler et al. 2005, Thacker and Starnes 2003, Usher et al. 2004a, 2004b, Webb and Maas 2002). At least one strain that is closely related to the marine cyanobacteria *Prochlorococcus* and *Synechococcus* belong to the sponge-specific microbial community (Hentschel et al. 2002) and was found geographically widely distributed in more than 20 different demosponges including the species *A. aerophoba* and *A. archeri* (Diaz et al. 1997, Steindler et al. 2005). Because of these extensive reference data this sponge-specific cyanobacterial strain was

chosen to test for cospeciation with *Aplysina* sponges. As the cyanobacterial data were not included in any of the previous chapters, they are discussed here in more detail.

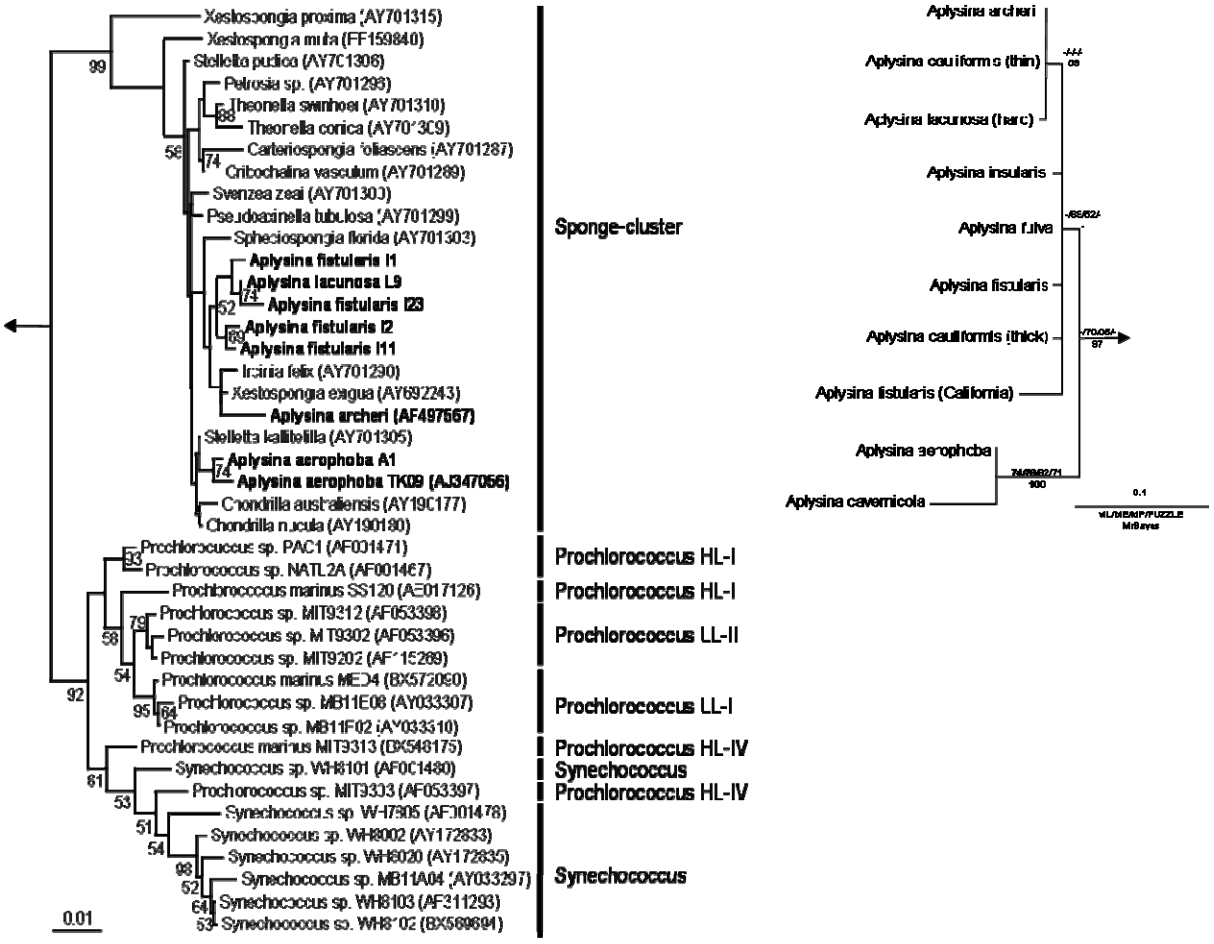

Fig. 1: Comparison of 16S rDNA *Synechococcus*-like cyanobacterial phylogeny (left) and ITS-2 *Aplysina* phylogeny (right). High-light (HL) and low-light (LL) adapted *Prochlorococcus* ecotypes and the sponge cluster are indicated. Host phylogeny is taken partly from Fig. 3, chapter 2. Scale bars show 1% and 10% sequence divergence in cyanobacterial and host tree, respectively.

Aplysina sponges were screened by PCR for the presence of cyanobacteria. Sequences from *A. aerophoba*, *A. fistularis*, and *A. lacunosa* (hard morphotype) together with additional sequences derived from *A. aerophoba*, *A. archeri*, other sponge species, and sequences of free marine *Prochlorococcus* and *Synechococcus* strains were used for the construction of a phylogenetic tree (Fig. 1). The tree is divided into two well supported clusters with the first containing exclusively sponge derived sequences (in-cluster similarity: 95.4-99.9%) and the

second containing only sequences of free marine cyanobacteria (in-cluster similarity: 95.3-99.7%). The clusters are up to 96% similar. Within the sponge-cluster resolution is low as indicated by multifurcations and low bootstrap values. *Aplysina* derived sequences are 97.5-99.4% similar but because of sequencing ambiguities the true similarity might be higher. The two *A. aerophoba* derived sequences that cluster together in the tree, probably represent the same cyanobacterium whereas the four *A. fistularis* derived sequences might represent at least two closely related cyanobacterial strains. The latter four sequences cluster together with the sequence from *A. cauliformis* and this group is one of the few that is supported albeit with low bootstrap values. However, the sequences from *A. aerophoba* and *A. archeri* tend to cluster with sequences from other, non-*Aplysina* sponges (see also tree by Steindler et al. 2005) and it seems therefore that the position of *Aplysina* derived sequences in the tree is not in agreement with cospeciation. Similarly, the phylotypes of *Xestospongia muta*, *X. proxima*, and *X. exigua* that are less than 98% identical have clearly distinct and distant positions in the tree and do also not support the idea of cospeciation. However, because of the lack of resolution a more variable marker should be used in further studies to unambiguously resolve relationships between sponge derived cyanobacterial sequences.

Interestingly, each of the congeneric pairs of *Chondrilla australiensis* and *C. nucula*, *Stelletta kallitetilla* and *S. pudica*, and *Theonella swinhoei* and *T. conica* contains highly similar cyanobacterial phylotypes (>99% similarity) indicating that closely related strains are present in closely related sponges of these three genera. However, in the phylogenetic tree (Fig. 1) only the close relationship of the *Theonella* spp. associated cyanobacteria is supported. This cluster is noteworthy as *T. swinhoei* and *T. conica* have different geographical distributions in contrast to *C. australiensis* and *C. nucula* as well as *S. kallitetilla* and *S. pudica* that each are coexisting in the same geographical region. The presence of highly similar cyanobacteria within closely related species might therefore not be the result of host species distribution.

Cyanobacteria closely related to *Synechococcus* and *Prochlorococcus* were shown to be vertically transmitted via sponge reproductive stages (chapter 6; Oren et al. 2005). However, Usher et al. (2001) found in a TEM study that only 25% of all investigated oocytes of *C. australiensis* contained cyanobacteria. The authors suggested that larvae that are free of cyanobacteria may acquire them horizontally from the water column after metamorphosis and establishing of a canal system for feeding (Usher et al. 2001). To date, the only example of cospeciation between sponges and bacteria was proposed for *Dysidea* species and *Oscillatoria*

spongeliae strains (Ridley et al. 2005, Thacker and Starnes 2003). In contrast to bacteriosponges that contain a diverse microbial community the *Dysidea – Oscillatoria* association resembles more the one host – one/ few symbionts systems of i.e., chemoautotrophs and clams, mussels or worms (Fisher et al. 1990). *Oscillatoria spongeliae* phylotypes were shown to exhibit a high degree of host specificity. However, at least one host switching event must have occurred as indicated by the comparison of host and cyanobacterial phylogeny. If vertical transmission is not a strict process and occasional horizontal bacterial transfer or host switching events occur the pattern of cospeciation is obscured as it seems to be the case for *Synechococcus*-like cyanobacteria in bacteriosponges. However, the close relationship of sponge associated cyanobacteria that is evident by a very well supported monophyletic cluster is striking (Fig. 1; Steindler et al. 2005). The next relatives, the free marine cyanobacteria *Prochlorococcus* and *Synechococcus* are genetically also closely related (>95% similarity), yet ecologically distinct populations have been described that are adapted to different niches along a depth gradient and are geographically widely distributed (Ferris and Palenik 1998, Rocap et al. 2002). The phylotypes of the sponge-cluster can be interpreted as a third ecotype that is specifically adapted to the niche sponge.

In summary, cospeciation of *Aplysina* sponges and probably other bacteriosponges with *Synechococcus*-like cyanobacteria appears unlikely although a close and specific relationship exists. Due to possible occasional environmental acquisition this might also be true for other sponge specific microbial lineages.

II. Vertical microbial transmission in bacteriosponges

Microscopic survey of sponge reproductive stages

The presence of microorganisms in adult tissue and reproductive material of each of 10 different sponge species was investigated by transmission electron microscopy (chapters 3, 4, and 6). The adult mesohyl of the five viviparous species *Callyspongia vaginalis*, *Mycale laxissima*, *Niphates digitalis*, *Tedania ignis*, and *Ulosa ruetzleri* was completely devoid of microorganisms and therefore these five species belong to the group of low-microbial abundance sponges. Microorganisms could also not be detected in the respective larvae. In contrast, the adult mesohyl of the sponges *Agelas wiedenmayeri*, *Aka coralliphagum*,

Ectyoplasia ferox, *Ircinia felix*, and *Smenospongia aurea* harbored large amounts of extracellular microorganisms. Accordingly, these species are high-microbial abundance sponges. *A. wiedenmayeri* and *E. ferox* are oviparous species that release oocytes or zygotes into the water column. Microorganisms were located predominantly in the peripheral region and were surrounded by a membrane in *A. wiedenmayeri* whereas no such membranes could be detected in *E. ferox*. *A. coralliphagum*, *I. felix*, and *S. aurea* are viviparous species that brood embryos and release larvae into the surrounding water. Microorganisms were present in the central region of the larvae. The microbial community in adult sponges and their respective reproductive material was found to be diverse in size, shape and membrane structures. Previous electron microscopical studies described at least seven different morphotypes in adult sponges (Friedrich et al. 1999, Vacelet 1975, Wilkinson 1978b), of which the three most abundant ones were found again in the adult mesohyl and in the reproductive stages of *A. wiedenmayeri*, *A. coralliphagum*, *E. ferox*, *I. felix*, and *S. aurea*. Therefore, a morphologically complex, sponge-specific microbial community rather than a single lineage is vertically transmitted.

There is a clear correlation between the presence of microorganisms in adult sponges and in the respective reproductive stages. All high-microbial abundance sponges contain microbes in their oocytes and larvae whereas the larvae of low- microbial abundance sponges are always devoid of microorganisms. The presence of microorganisms in reproductive stages was previously shown for other bacteriosponges (see chapter 1, table 1) and these cumulative data strongly suggest that microorganisms in high-microbial abundance sponges are vertically transmitted. In this Ph.D. thesis, the presence of microbes in reproductive stages of species within the orders Agelasida (*A. wiedenmayeri*), Haplosclerida (*A. coralliphagum*), and Poecilosclerida (*E. ferox*) was shown for the first time. In total, bacteriosponges of at least nine orders are now known to contain microbes in their reproductive stages including oviparous as well as viviparous species (chapter 1, table 1; chapters 3 and 6). Neither the host phylogeny nor the mode of reproduction seems to be a determining factor and vertical transmission is apparently a common and widespread mechanism among high-microbial abundance sponges.

Vertical transmission – *Ircinia felix* as a model system

Based on the results of the electron microscopy survey the viviparous sponge *I. felix* was chosen for a detailed documentation of vertical transmission (chapters 3, 4, and 5). Electron microscopy was applied for a more precise localization of microbes within the larvae. The larval outer region was found to be almost devoid of microorganisms. When present, single microbes were located extracellularly between the epithelium sponge cells. In contrast, the central region contained large amounts of morphologically diverse microorganisms which were located extracellularly around the archaeocytes. A similar gradient in microbial distribution in larvae was described for the microbial community of *Ircinia oros* as well as of other bacteriosponges (Ereskovsky and Tokina 2004, Kaye and Reiswig 1991) and for cyanobacteria in *Diacarnus erythraenus* (Oren et al. 2005). The fact that microbes are present within sponge oocytes, embryos and larvae and are not attached to the surface of reproductive stages implies that these microorganisms represent maternal mesohyl microbes that were taken up during gametogenesis and embryogenesis and do not represent seawater contaminations affiliated with reproductive stages by accident.

Microorganisms were also localized in newly grown *I. felix* juvenile sponges 1-3 days post settlement. The presence of choanocyte chambers demonstrates that metamorphosis and rearrangement of the body structure was already accomplished and the juveniles were able of pumping water and filtering food particles from the water column. Diverse microorganisms were present in the space between sponge cells. Similarly, numerous different microbes could be observed in *I. strobilina* and *S. aurea* juvenile sponges that were either equally scattered between sponge cells (*I. strobilina*) or grouped around sponge cells (*S. aurea*) (Fig. 2A, C). Choanocyte chambers could also be found in *I. strobilina* also showing the presence of a functional aquiferous system in this species (Fig. 2B). The morphological diversity of juvenile microbial communities resembles that of adult and larval sponges indicating the presence of the same microbial community that was described from adult and larval specimens.

Based on electron microscopy data, the vertical transmission of a complex microbial community from adult over larval to juvenile stages could be shown for the species *I. felix*. In general, the comprehensive electron microscopical data of this Ph.D. thesis and previous studies provide morphological evidence that vertical transmission of microbial communities is a common and widespread phenomenon in bacteriosponges and is probably the important mechanism by which specific sponge – microbiota relationships are maintained.

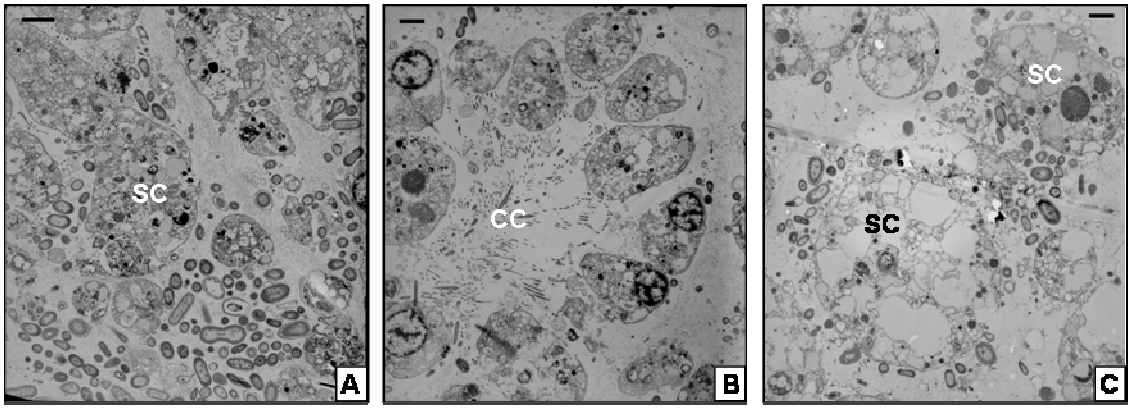

Fig. 2: Transmission electron microscopy of *Ircinia strobilina* juvenile mesohyl (A) and choanocyte chamber (B) as well as of *Smenospongia aurea* juvenile mesohyl (C). CC: Choanocyte chamber, SC: sponge cell. Scale bars: 1µm (A), 2 µm (B, C).

Denaturing gradient gel electrophoresis (DGGE) was applied to determine and compare the microbial profiles of adult, larvae and juvenile samples of *I. felix*. The banding pattern of adult and larvae were similar suggesting that phylogenetically similar microbial communities are present in adult sponges and respective larvae. The juveniles shared about half of all bands with adult and larvae samples demonstrating that at least half of the total adult microbial community is vertically transmitted.

Larvae pools released by the same adult individual as well as larvae pools released by different adult individuals were also analyzed (chapter 4). The bacterial profiles of larvae from adult #4 were similar sharing almost 75% of their bands. Apparently, these larvae transfer similar subsets of the adult microbial community. In contrast, bacterial profiles from larvae of adult #1, #2, #4, and #5 were found to be more variable and similarities ranged down to 49%. This is a surprising finding as the variability within a species was thought to be low (Taylor et al. 2004). Assuming that the incorporation of microbes into early reproductive stages might occur by chance instead of being a selective uptake of specific lineages this would explain the fine-scale intraspecies variations. It would be intriguing to follow up these differences in juvenile and growing adult sponges to see whether intraspecies variations are vanishing or are still present in adult *I. felix*.

DGGE analysis revealed that a large proportion of the adult microbial community in *I. felix* rather than single phylogenetic lineages are vertically transmitted through larvae to juvenile sponges. These molecular data are in agreement with the electron microscopy results of *I. felix* and other bacteriosponges. Interestingly, intraspecies differences in the microbial

community of larvae released by different adult sponges were observed whereas larvae released by the same adult sponge contained similar microbial communities.

Phylogenetic evidence for vertical transmission

At the beginning of this Ph.D. thesis no phylogenetic information about microorganisms in oocytes, embryos, larvae, and juvenile sponges was available. It was therefore one major aim of this thesis to phylogenetically identify the microbes within sponge reproductive stages as well as within juvenile sponges and to confirm vertical transmission of a diverse sponge-specific community as was suggested by electron microscopy and DGGE data.

In total, 117 sequences were obtained from adult samples of the sponges *E. ferox*, *I. felix*, *S. aurea* and *X. muta* (chapters 4 and 6). The total microbial diversity in these sponges was found to be high and included phylotypes of at least five eubacterial phyla. Many of the sequences of this Ph.D. thesis are most similar to other bacteriosponge derived sequences and it can be concluded that the four species are high-microbial abundance sponges that contain the specific microbial community. Almost 200 sequences were obtained from reproductive stages of *A. wiedenmayeri*, *E. ferox, I. felix*, *S. aurea*, and *X. muta* as well as of *I. felix* and *S. aurea* juveniles. The overall diversity is as high as in adult sponges and sponge specific phylotypes of seven of the nine previously reported phyla were found with the exception of Nitrospira and Poribacteria (Hentschel et al. 2006). The latter would not have been expected in this study because of differences in the primer regions. While all sequences from *S. aurea* juveniles are closely related to other sequences from sponges more than half of all *I. felix* juvenile sequences are similar to environmental sequences. This is in accordance with the DGGE banding pattern that indicated a mixture of sponge specific microbes and seawater bacteria due to an artefact in DNA extraction. These sequence data demonstrate that a phylogenetically diverse microbial community is present in sponge reproductive stages and this community mainly resembles the diversity in adult sponges.

Phylogenetic trees were constructed with the sequences obtained in this Ph.D. thesis and additional reference sequences (chapters 4 and 6). Vertical transmission clusters (VT-clusters) were defined as monophyletic clusters of both adult and offspring derived sequences. From this comprehensive analysis, a total of 23 VT-clusters were detected that were distributed in 8 different eubacterial phyla. Additionally, sequences that could not be affiliated with any known eubacterial phylum and might represent a novel bacterial lineage also formed a VT-

cluster. Remarkably, 25% of all VT-clusters are double or triple VT-clusters because they contain adult and offspring derived sequences from each of two or even three species. Obviously, the same phylotypes are vertically transferred in different bacteriosponges.

92% of all VT-clusters contained at least one additional sponge derived sequence and 75% of all VT-clusters are identical with the previously identified sponge specific clusters (Hentschel et al. 2002). Apparently, vertical transmission is a highly specific process in which exclusively the members of the sponge specific microbial community are passed on to the next generation via the reproductive stages. At the same time vertical transmission seems to be unselective as no obvious differences in the transmission of individual sponge specific lineages could be observed. This might be due to the simple anatomy of sponges. Because of the lack of reproductive organs gametogenesis and embryogenesis occur in the mesohyl. And while microorganisms in other invertebrate hosts are defined to specialized cells (bacteriocytes, e.g. in many insect hosts and deep-sea clams) or to certain organs (e.g. light organ of squids, trophosome tissue of vestimentiferan tube worms) microbes in sponges are located freely in the mesohyl. Accordingly, reproduction occurs in close proximity to microbes and it would be conceivable that these microbes that are present at the site of reproduction are incorporated into early reproductive stages and are vertically transmitted. Therefore, the vertical transmission of certain lineages might be somewhat random. However, electron micrographs as well as fluorescence *in situ* hybridization analysis indicated that the microbial community in the mesohyl is well mixed (Fieseler et al. 2004, Friedrich et al. 1999, Vacelet 1975). This might be the reason that many different microbes are incorporated into reproductive stages and thereby assure that a large fraction of the adult microbial diversity is vertically transmitted.

These phylogenetic data are in agreement with the electron microscopy and DGGE (in the case of *I. felix*) results of this Ph.D. thesis in that (i) a diverse microbial community rather than single lineages are vertically transmitted, (ii) specifically members of the sponge specific microbial community are vertically transmitted, (iii) vertical transmission is a common and widespread mechanism among bacteriosponges, and (iv) vertical transmission is independent of sponge phylogeny, sponge geographic distribution, and mode of reproduction. In conclusion, vertical transmission is clearly important for the establishment and maintenance of the phylogenetically complex yet highly sponge specific microbial communities of bacteriosponges.

III. Vertical transmission but lack of cospeciation – a paradox?

The results of this Ph.D. thesis clearly demonstrated that a large subset of the highly diverse adult microbial community of bacteriosponges is vertically transmitted through sponge reproductive stages. However, phylogenetic congruence of *Aplysina* sponges and *Synechococcus*-like cyanobacterial strains was not detected and cospeciation of other sponge specific lineages also appears unlikely. A possible explanation might be an occasional infidelity of vertical transmission. If the incorporation of microbes into early reproductive stages occurs unselective as indicated by the phylogenetic results of this Ph.D. thesis not every lineage is always vertically transmitted and the pattern of cospeciation would be obscured. Alternatively, sponge specific associates might sometimes be acquired horizontally from other sponge individuals. Such host switching events that were already described for the two-partner system of *Dysidea* sponges and *Oscillatoria spongeliae* strains (Ridley et al. 2005, Thacker and Starnes 2003) might also have occurred in bacteriosponges. A third possibility might be small amounts of horizontal acquisition of microorganisms from seawater. Recently, feeding experiments with microbial seawater populations and sponge specific microbial communities that were physically separated from their host sponges were performed (Wehrl et al. 2007). The uptake rates of sponge specific microbes were low in bacteriosponges and significantly reduced by almost two orders of magnitude compared to uptake rates of seawater microbes. However, these data would allow occasional horizontal transfer of sponge specific microbes. Occasional infidelity of vertical transmission as well as host switching events and small amounts of horizontal acquisition are likely to occur in bacteriosponges and would cause the lack of cospeciation that was observed in this Ph.D. thesis. The strategy of maternal transmission of the specific microbial community in bacteriosponges clearly represents a bottleneck for the specificity and maintenance of the association yet it seems to be "wide open".

Vertical microbial transmission and congruence of host and symbiont phylogeny implying cospeciation seems to be a widespread phenomenon among terrestrial arthropods and was reported e.g. for symbionts and aphids (Clark et al. 2000), carpenter ants (Sauer et al. 2000), cockroaches (Bandi et al. 1995), and tsetse flies (Chen et al. 1999). In contrast to bacteriosponges, these hosts are usually associated with one primary microbial symbiont that resides in specialized host cells (bacteriocytes). It is thought that these systems originated from a single ancient infection and subsequently diverged in parallel for millions of years

(Moran and Baumann 2000). In the marine environment, vertical transmission of symbionts has also been described for many invertebrate hosts including e.g. ascidians (Hirose and Fukuda 2006), bryozoans (Lim and Haygood 2004), and annelids (Giere et al. 1991). However, to my knowledge cospeciation was only identified for vesicomyid clams and chemoautotrophic symbionts (Peek et al. 1998). Despite widespread vertical transmission of associated microorganisms the lack of cospeciation might be the rule rather than the exception in the marine environment. The reasons for that are unknown but methodological aspects (the lack of robust phylogenies) as well as ecological aspects (water as surrounding medium) might be important.

IV. Ecological and evolutionary implications of vertical transmission

Ecological implications

The microbial community in bacteriosponges is phylogenetically highly diverse (Hentschel et al. 2006) and comprises probably a wide range of physiological properties. Assuming that microbial functions are beneficial to the host sponges (e.g. the provision of photosynthetic products (Wilkinson and Fay 1979) or the production of bioactive compounds as chemical defence against sponge predators (Hill 2004)) it would be very important to assure their presence in the next generation. The transfer of a complex microbial community via reproductive stages in various bacteriosponges as documented in this Ph.D. thesis strongly suggests that vertical transmission is the commonly used strategy to maintain the phylogenetically and probably physiological diversity of microbes in adult sponges. Moreover, also the larvae may benefit from the presence of microbes within their central cavity. During this critical non-feeding life stage the provision of nutritional products and energy by microbes may be highly important in successfully competing with other sessile organisms for a suitable substratum for settlement. It might also be conceivable that microbes produce bioactive secondary metabolites that prevent the sponge larvae from being digested by predators. This has been shown for the bryozoan *Bugula neritina* of which the larvae are protected by a symbiont produced cytotoxin (Lopanik et al. 2004).

Interestingly, no selectivity in the transmission of specific phylogenetic lineages could be observed and the microbial incorporation into early reproductive stages appears to occur somewhat at random. Generally, a sponge might be considered as a habitat consisting of

several micro-niches restricted to specific microbial physiological abilities. For example, the oxidation of ammonium which is the first step in the nitrification process is catalyzed by betaproteobacterial strains *Nitrosospira* spp. and *Nitrosomonas* spp. as well as by gammaproteobacterial *Nitrosococcus* spp.. Additionally, also some archaea are able of oxidizing ammonium (Könneke et al. 2005). 16S rDNA sequence data indicate that representatives of all four groups are present in sponges (Bayer et al. submitted, in prep, Diaz et al. 2004, Hentschel et al. 2002). However, to maintain the process of ammonium oxidation in the next generation it would be enough to transmitted one member of these four groups. In other words, if it is more important that every ecological niche in the sponge is occupied than that every microbial lineage is present in the next generation then it might be sufficient to transmit a large proportion of the adult microbial community as seen in this Ph.D. thesis.
It is currently unknown if the nature of the sponge – microbe association is commensal or mutualistic. However, several findings strongly indicate a closely evolved and coadapted system. The microbial community in bacteriosponges has been described to be permanent, stable and highly specific (Hentschel et al. 2006). Sponge specific microbes were neither detected in seawater nor could they be isolated and cultivated so far. The beneficial involvement of microbes in the sponge metabolism seems to be very likely (Bayer et al. submitted, Wilkinson and Fay 1979). The results of this Ph.D. thesis revealed that the sponge specific microbial community is vertically transmitted in bacteriosponges. Despite the lack of clear evidence of coadaptation all these results point to a mutualistic association with benefits for the host sponges as well as for the associated microbes.

Evolutionary implications

The association between sponges and microorganisms is generally thought to be ancient. It is conceivable that one or several microbial infection events might have occurred in the Precambrian when sponges first appeared and before sponge radiation and geographical expansion took place ("Precambrian acquisition hypothesis") (Wilkinson 1984). A close association would have separated these microbes from seawater microbes and subsequent strict vertical microbial transmission for millions of years would explain the presence of similar strains in phylogenetically and geographically distinct sponges. However, sequence analysis studies revealed that most sponge specific phylotypes are not deeply branching in phylogenetic trees but have separated more recently from their free-living relatives (Hentschel et al. 2002). Moreover, the existence of low-microbial abundance sponges would imply independent loss of associates in many sponges within several sponge orders. This might be a

conceivable scenario in sponges that invaded into freshwater habitats and lost their microbes possibly due to an obligate requirement of sodium ions by the associated bacteria (Wilkinson 1978a, Wilkinson 1984) but the reasons are less clear for marine sponges co-occurring in the same habitat as high-microbial abundance sponges.

The results of this Ph.D. thesis strongly suggest that vertical transmission is the major mechanism for establishing and maintaining specific microbial communities in bacteriosponges. The data also indicate that vertical transmission is not a strict process and occasional horizontal transfer of sponge specific microbes may also occur. Because of such a "leaky" system of vertical microbial transmission the association of microorganisms and sponges is not a closed but an open system with some environmental exchange. Theoretically, this would be consistent with an evolutionarily ancient origin of the association between sponges and microorganism but would also be consistent with the lack of ancient sponge specific microbes in phylogenetic trees. In conclusion, the documentation of vertical transmission points to a long-term association rather than a recent infection event.

V. Directions for future research

This Ph.D. thesis gives a comprehensive overview of vertical transmission of microorganisms in several Caribbean bacteriosponges. Future studies might concentrate on the mechanistic aspects such as the early and late processes of the transmission. In particular it would be interesting to determine the mechanisms and the time point of microbial incorporation into early reproductive stages using molecular methods such as fluorescence *in situ* hybridization. Electron microscopy pictures of this thesis as well as other publications showed that vertical microbial transmission also includes intracellular steps possibly mediated by phygocytosis. It is entirely unknown how the microorganisms become extracellular again in later reproductive stages such as embryo and larvae. To address this question a combination of visual and molecular methods should be applied.

In this Ph.D. thesis settlement *in situ* experiments were performed on tropical reefs to document vertical transmission of microbes from adult over larvae to juvenile sponges. These experiments could be repeated under controlled laboratory conditions and the influence of varying factors on vertical transmission and the subsequent establishment of microbes in the

juvenile sponges could be assessed. A possible experimental design might be to keep the larvae and juveniles in sterile seawater and to investigate whether the microbial numbers and diversity remains stable or changes over time. A decrease in amount and diversity would be noteworthy as it indicates that sponges might be able of digesting their own microbial associates. An experimental perturbation could be the treatment with antibiotics. It would be of interest to obtain bacteria-free larvae or juvenile sponges. Subsequent colonizing studies and parallel sponge gene expression studies would provide fundamental insights into the mechanisms of the sponge – microbe association as well as in host developmental processes.

This Ph.D. thesis focused on the eubacterial community in sponges. However, the presence of archaea in sponges has long been recognized (Preston et al. 1996, Webster et al. 2001) and the association of *Cenarchaeum symbiosum* with *Axinella* sponges is regarded to be specific and consistent (Holmes and Blanch 2006, Margot et al. 2002). It appears likely that also archaeal members of the sponge associated microbial community are vertically transmitted and future studies should address this question.

Interesting observations of this study were the lack of selectivity in the transmission of sponge specific phylotypes and the differences in the microbial DGGE-profiles of larvae released by different adult individuals. To further investigate intraspecies variability of sponge associated microbial communities the presence in adult sponges, vertical transmission, and putative horizontal uptake of specific microbial lineages should be documented for a statistical sufficient number of sponge individuals and over a long-term period of several years. Possible candidates could be the intensively studied *Synechococcus*-related cyanobacterial associate (Oren et al. 2005, Steindler et al. 2005, Usher et al. 2001), members of the exclusively in sponges present candidate phylum Poribacteria (Fieseler et al. 2004), or microorganisms that might be involved in the sponge metabolism such as nitrifying bacteria and archaea (Bayer et al. submitted, Diaz et al. 2004).

Finally, although the results of this study indicate that cospeciation of many sponge specific microbes with bacteriosponges is unlikely it might be worth while to test whether Poribacteria have cospeciated with *Aplysina* sponges. The phylogeny of eight *Aplysina* species was established in this Ph.D. thesis (chapter 3) and the presence of poribacterial phylotypes in these species was documented previously (Fieseler et al. 2004). The fact, that so far the

candidate phylum Poribacteria was not detected in seawater, sediments or other marine invertebrates might allow a strict vertical transmission and accordingly, cospeciation.

VI. References

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(3) **Schmitt S**, Wehrl M, Bayer K, Siegl A, Hentschel U. (2007) Marine sponges as models for commensal microbe-host interactions. Symbiosis. in press.

(4) **Schmitt S**, Wehrl M, Lindquist N, Weisz J, Hentschel U. Vertical transmission of microorganisms in Caribbean reef sponges. Proceedings of the 7th International Sponge Symposium (ISS). submitted.

(5) Bayer K, **Schmitt S**, Hentschel U. Microbial nitrification in Mediterranean sponges: Possible involvement of ammonium-oxidizing *Betaproteobacteria*. Proceedings of the 7th International Sponge Symposium (ISS). submitted.

(6) Bayer K, Siegl A, **Schmitt S**, Hoffmann F, Hentschel U. Unravelling microbial diversity and metabolism in marine sponges. Proceedings of the Leopoldina Symposium 2006 "Life strategies of microorganisms in the environment and in host organisms". submitted.

(7) **Schmitt S**, Angermeier H, Schiller R, Weisz JB, Lindquist N, Hentschel U.Comprehensive phylogenetic analysis reveals the vertical transmission of a uniform yet phylogenetically complex microbial community in six bacteriosponges. in preparation.

(8) Bayer K, **Schmitt S**, Hentschel U. Microbial nitrification in the marine sponge *Aplysina aerophoba* (Verongida, Demospongiae). in preparation.

Conferences

(1) 11th International Symposium on Microbial Ecology (ISME), Vienna, Austria (August 2006)

Oral: Vertical transmission of a complex microbial consortium in the sponge *Ircinia felix*

Oral: Microbial diversity, symbiosis and biotechnological potential of marine sponges (contribution to the roundtable discussion: Marine sponges as microbial fermenters)

(2) 5th International Symbiosis Society (ISS) Symposium, Vienna, Austria (August 2006)

Oral: Vertical transmission of a complex microbial consortium in the sponge *Ircinia felix* (funded by a travel grant from the "Jubiläumsstiftung zum 400-jährigen Bestehen der Universität Würzburg")

(3) 7th International Sponge Symposium (ISS), Buzios, Brasil (May 2006)

Oral: Vertical transmission of bacteria in Caribbean reef sponges

Poster: S. Schmitt, U. Hentschel, S. Zea, T. Dandekar, M. Wolf

ITS-2 and 18S rRNA gene phylogeny of Aplysinidae

Poster: K. Bayer, **S. Schmitt**, U. Hentschel

Microbial nitrification in *Aplysina aerophoba*

(4) Evolutionary Chemical Ecology – Meeting of the graduates of the German Zoological Society, Wuerzburg, Germany (February 2005)

Oral: Marine sponges as ancient niches to bacteria: concerted evolution or niche adaptation?

Workshops

(1) **Getting funded - Proposal Writing.** Workshop of the Graduate School "Infektionsforschung" in English (BioScript International, Dr. R. Willmott), 22.03.-23.03.2007

(2) **Projektmanagement.** Workshop of the Graduate School "Infektionsforschung" (Einszeit Managementberatung und Coaching, S. Dierig), 01.03.-02.03.2007

(3) **Poster Design and Presentation.** Workshop of the Graduate School "Infektionsforschung" in English (BioScript International, Dr. R. Willmott), 07.09.-08.09.2006

(4) **Effective Scientific Writing.** Workshop of the Graduate School "Infektionsforschung" in English (BioScript International, Dr. R. Willmott), 04.09.-06.09.2006

(5) **Different applications of light and electron microscopy (CLSM, REM, TEM).** University of Wuerzburg, Department of Cell and Developmental Biology (Prof. G. Krohne), 16.02.-27.02.2004

(6) **Methods in molecular sequence analysis and phylogenetic tree construction (Align, Bioedit, ClustalX, Phylip, PAUP, MrBayes).** University of Wuerzburg, Department of Bioinformatics (Dr. M. Wolf), 03.11.-07.11.2003

Field trips

(1) **Deep-sea expedition to the Fiji/Lau Basin**
3-weeks expedition, May 2005
P.I.: R. C. Vrijenhoek (Monterey Bay Aquarium Research Institute)

(2) **NOAA - National Undersea Research Center (NURC), Key Largo, FL, USA**
2 to 3-weeks expeditions, September 2003, May and August 2004
P.I.: N. Lindquist (University of North Carolina at Chapel Hill, USA)

(3) **HYDRA Institut für Meereswissenschaften, Fetovaia, Elba, Italy**
2-weeks excursion "Marine Lebensräume", August 2001

(4) **Ruđer Bošković Institute, Rovinj, Croatia**
2-weeks expedition, July 2001
P.I.: U. Hentschel (Research Institute for Infectious Diseases)

(5) **Laboratoire Arago, Banyuls-sur-Mer, France**
2-weeks expedition, April 2001
P.I.: U. Hentschel (Research Institute for Infectious Diseases)

Curriculum Vitae

Susanne Schmitt

Personal data

Date of birth	23 December 1977
Place of birth	Alzenau, Germany
Nationality	German

School and University Education

since 2003	Ph.D. thesis: "Vertical microbial transmission in Caribbean bacteriosponges"
	Julius-Maximilians-University of Wuerzburg, Germany
	Group leader: PD Dr. U. Hentschel
2002	Diplom (equivalent to MSc) in Biology
	Julius-Maximilians-University of Wuerzburg, Germany
	Major in Microbiology, Ecology, Palaeontology
1997	Abitur (equivalent to 'A-level')
	Kronberg Gymnasium Aschaffenburg, Germany

Memberships

since 2006	International Symbiosis Society
since 2005	Graduate school "Infektionsforschung", University of Wuerzburg, Germany

Grant

2006	Travel grant
	"Jubiläumsstiftung zum 400-jährigen Bestehen der Universität Würzburg"