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Sponge white patch disease affecting the Caribbean sponge *Amphimedon compressa*

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ABSTRACT: We report on a novel sponge disease, hereafter termed 'sponge white patch' (SWP), affecting the Caribbean sponge species *Amphimedon compressa*. SWP is characterized by distinctive white patches of variable size that are found irregularly on the branches of diseased sponges. Nearly 20% of the population of *A. compressa* at Dry Rocks Reef, Florida, USA, showed symptoms of SWP at the time of investigation (November 2007–July 2010). Approximately 21% of the biomass of SWP individuals was bleached, as determined by volume displacement. Scanning electron microscopy analysis showed severe degradation of bleached tissues. Transmission electron microscopy of the same tissues revealed the presence of a spongin-boring bacterial morphotype that had previously been implicated in sponge disease (Webster et al. 2002; Mar Ecol Prog Ser 232:305–309). This particular morphotype was identified in 8 of 9 diseased *A. compressa* individuals investigated in this study. A close relative of the aforementioned disease-causing alphaproteobacterium was also isolated from bleached tissues of *A. compressa*. However, whether the spongin-boring bacteria are true pathogens or merely opportunistic colonizers remains to be investigated. Molecular fingerprinting by denaturing gradient gel electrophoresis (DGGE) demonstrated a distinct shift from the microbiota of healthy *A. compressa* to a heterogeneous mixture of environmental bacteria, including several phylotypes previously implicated in sponge stress or coral disease. Nevertheless, tissue transplantation experiments conducted in the field failed to demonstrate infectivity from diseased to healthy sponges, leaving the cause of SWP in *A. compressa* to be identified.

KEY WORDS: Porifera · Spongin-boring bacterium · Coral reef disease · Pathogenesis · Alphaproteobacterium

INTRODUCTION

Marine sponges (phylum Porifera) are among the most ancient multicellular animals (metazoans), with a fossil record dating back >630 million years (Love et al. 2009). Sponges are known for their interactions with various types of microorganisms, including viruses, bacteria, cyanobacteria, archaea, fungi, protozoa and single-celled algae (Hentschel et al. 2006, Taylor et al. 2007, Webster & Taylor 2012). In general, these microorganisms serve as food particles which are retained from seawater in the choanocyte chambers, translocated into the mesohyl interior and digested by phagocytosis. Many sponges, the so-called high microbial abundance (HMA) sponges or bacteriosponges (Reiswig 1981, Hentschel et al. 2003), contain symbiotic microbial consortia within their mesohyl matrix that may amount to nearly 40% of their biomass (Vacelet 1975). To date, more than 17 different bacterial phyla and 12 candidate phyla have been discovered from sponges, with the vast majority of these microbes defying attempts at labo-

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ratory cultivation (Webster et al. 2010, Schmitt et al. 2012, Simister et al. 2012).

As has been reported for corals and other marine invertebrates (Webster 2007), sponges can also suffer from diseases and bleaching. Disease development typically starts with the appearance of discolored patches, followed by tissue disintegration, leading to the exposure of the sponge skeleton. Sponge diseases have thus far been reported from many geographic regions, including the Great Barrier Reef, the Indo-Pacific, the Mediterranean and the Caribbean (Harvell et al. 1999). Sponge diseases that have already been reported include *Aplysina* red band syndrome (ARBS) (Olson et al. 2006), *Aplysina* black patch syndrome (Webster et al. 2008), sponge orange band (SOB) disease of *Xestospongia muta* (Cowart et al. 2006, López-Legentil et al. 2010, Angermeier et al. 2011), brown lesion necrosis or disease-like syndrome of *Ianthella basta* (Cervino et al. 2006, Luter et al. 2010), spongin-boring necrosis of *Rhopaloides odorabile* (Webster et al. 2002) and pustule disease of *Ircinia fasciculata* and *I. variabilis* (Maldonado et al. 2010). Outbreaks of various sponge diseases have indeed proven to be fatal on several occasions in different localities (Gaino et al. 1992, Vacelet et al. 1994, Maldonado et al. 2010, Cebrian et al. 2011). Research on sponge diseases has generally been hampered by the fact that the death of any given sponge is often hard to define and frequently goes unnoticed.

The marine sponge *Amphimedon compressa*, commonly called the erect rope sponge, is found throughout Florida, USA, the Bahamas and the Caribbean (Zea et al. 2009). Individuals range up to 1 m in size and are characterized by a distinct red coloration. *A. compressa* has osculae scattered all over its surface, and its tissue is soft and flexible. *A. compressa* belongs to the low microbial abundance (LMA) category of sponges following the definition of Hentschel et al. (2003), which implies that the sponge mesohyl is visually devoid of microorganisms as judged by transmission electron microscopy and that bacterial numbers and phylogenetic composition are similar to those of seawater. Several secondary metabolites have been isolated from this species, comprising fatty acids (Carballeira et al. 1998), sterols (Ballantine & Williams 1977) and the alkaloid amphitoxin, which displays antibacterial activities as well as pronounced antifeedant activities against diverse reef fish (Albrizio et al. 1995, Thompson et al. 2010). In this study, we describe, to our knowledge for the first time, a pathological condition of the rope sponge *A. compressa*, which we name 'sponge white patch' (SWP) disease owing to the irregular white patches that cover the body of afflicted sponge individuals.

MATERIALS AND METHODS

Sponge collection

Samples of *Amphimedon compressa* (class Demospongiae, order Haplosclerida, family Niphatidae) were collected by SCUBA diving at a depth of 4–30 m within the Florida Keys National Marine Sanctuary at Dry Rocks Reef, Florida (25° 07′ 91″ N, 80° 17′ 56″ W) in November 2007 and at Conch Reef, Florida along a line transect (24° 56′ 86″ N, 80° 27′ 23″ W) in May and September 2009 and July 2010. Altogether, 709 individuals of *A. compressa*, grouped into either healthy (n = 538) or diseased categories (n = 171), were investigated in this study. The frequency of SWP-diseased individuals was determined for Dry Rocks Reef by inspecting 80 randomly selected 1 m^2 quadrats via SCUBA diving. The sponge samples collected for further experimental work were transferred to the surface in seawater-containing Ziploc bags and were kept cool until further processing within 1–2 h. The amount of white and red tissue per SWP individual (n = 40 individuals) was determined by volume displacement using a seawater-filled graduated plastic cylinder. For each sponge, the healthy tissue was dissected immediately upon return to the marine station from the diseased tissue prior to the analysis using a scalpel, and the volume of seawater displaced by each of the 2 fractions was determined independently.

Electron microscopy

Scanning electron microscopy (SEM) was performed on 0.5 cm^3 sections of sponge tissue from healthy as well as diseased (red and white) *A. compressa* specimens following established protocols by Angermeier et al. (2011). This involved the excision of sponge tissue, storage in 6.25% glutaraldehyde-phosphate-buffered solution, a 5× washing procedure with Soerensen-phosphate buffer (50 mM, pH 7.4) for 5 min each and dehydration in an increasing acetone series starting with 30% for 15 min, followed by 50% for 20 min, 75% for 30 min, 90% for 45 min and 6× incubation at 100% for 30 min. The samples were critical point dried in 100% acetone under pressure, sputtered with gold-palladium and

stored in the dehydrator until examination with the scanning electron microscope (Zeiss DSM 962). Transmission electron microscopy (TEM) was performed on the same *A. compressa* individuals as used for SEM following the procedure of Angermeier et al. (2011). The prepared samples were sectioned with an MT-7000 ultramicrotome (RMC) for examination with the transmission electron microscope (Zeiss EM10).

Denaturing gradient gel electrophoresis

For DNA extraction, 0.5 cm^3 sponge samples were preserved in 70% ethanol, air-dried and homogenized with a mortar and pestle in liquid nitrogen. The DNA extraction and 16S rRNA gene amplification for denaturing gradient gel electrophoresis (DGGE) were performed as described in Angermeier et al. (2011) with the primer pair 341f with GC-clamp and 907r (Muyzer et al. 1998), which resulted in a PCR fragment of approximately 566 bp in size. The PCR was conducted using a T3 thermocycler (Biometra) with the following conditions: initial denaturation at 94°C for 2 min, 34 cycles of denaturation at 94°C for 1 min, primer annealing at 57°C for 30 s followed by elongation at 72°C for 40 s and a final extension step at 72°C for 5 min. Thirty-four PCR cycles were necessary to obtain a clearly visible DGGE pattern for this LMA sponge. The size and quality of the obtained PCR products were examined on 2% agarose gels stained with ethidium bromide. DGGE was conducted on 10% (w/v) polyacrylamide gels with a denaturing gradient of 0–100% (Muyzer et al. 1998). Selected DGGE bands were excised with an ethanol-sterilized scalpel under UV light and DNA was eluted overnight with 25 µl H_2O at 4°C. The excised DGGE bands were re-amplified with the primer pair 341f and 907r using the following conditions: initial denaturation at 94°C for 2 min followed by 29 cycles of denaturation at 94°C for 1 min, annealing of the primers at 60°C for 30 s and elongation at 72°C for 40 s. The final extension step was at 72°C for 5 min.

The obtained PCR products were purified, ligated into the vector pGEM-T-Easy (Promega) and transformed into *Escherichia coli* Novablue cells. The plasmid DNA was isolated by standard miniprep procedures (Sambrook et al. 1989) and digested with *Eco*RI to confirm the correct insert size by agarose gel electrophoresis. Sequencing was conducted for one clone per DGGE band. Chimeras were identified with the program Pintail (Ashelford et al. 2005) by comparison against the 5 most closely related 16S ribosomal ribonucleic acid (rRNA) gene sequences from culturable bacteria and removed from the data set. All 16S rRNA gene sequences were deposited in GenBank (accession numbers HQ659567–HQ659575).

Infection experiments

Sponge tissue transplantation experiments were conducted along a 20 m transect at Conch Reef in September 2009. Healthy or diseased tissues (n = 10 for each group) were attached to an equivalent number of healthy, randomly selected individuals of *Amphimedon compressa* using cable ties. Infection experiments of healthy *A. compressa* were performed with alphaproteobacterial strain HA007 (accession number JQ582943), which was previously isolated from SWP *A. compressa* tissues and is closely related to alphaproteobacterium strain NW4327 (Webster et al. 2002). Ten milliliters of both broth culture and a 1:10 dilution of broth in sterile seawater (each n = 3) of this isolate were injected into healthy *A. compressa* specimens. ZoBell medium without bacterial inoculum served as negative control and broth culture grown from 10 pooled *A. compressa* white tissue patches served as positive control. The recipient sponges were monitored daily by SCUBA diving for up to 9 d, at which point the field work was terminated.

RESULTS AND DISCUSSION

We report herein a disease phenomenon affecting the sponge *Amphimedon compressa* from coral reefs off Key Largo, Florida, USA. The disease was observed in winter 2007 at Dry Rocks Reef and in summer 2009 and 2010 also at Conch Reef, both within the Florida Keys National Marine Sanctuary. We named the disease 'sponge white patch' owing to the large, distinct white patches that appear irregularly on diseased sponge individuals (Fig. 1a–c). A transition zone between red and white tissues or inner and outer regions was not observed; rather, the red and white tissues were sharply separated from each other (Fig. 1d).

To assess the impact of SWP disease on the population of *Amphimedon compressa* at Dry Rocks Reef, a quantitative survey was performed by counting healthy and diseased specimens. Of the 610 individuals of *A. compressa* surveyed, 82% (n = 503) was visually healthy and 18% (n = 107) showed the spe-

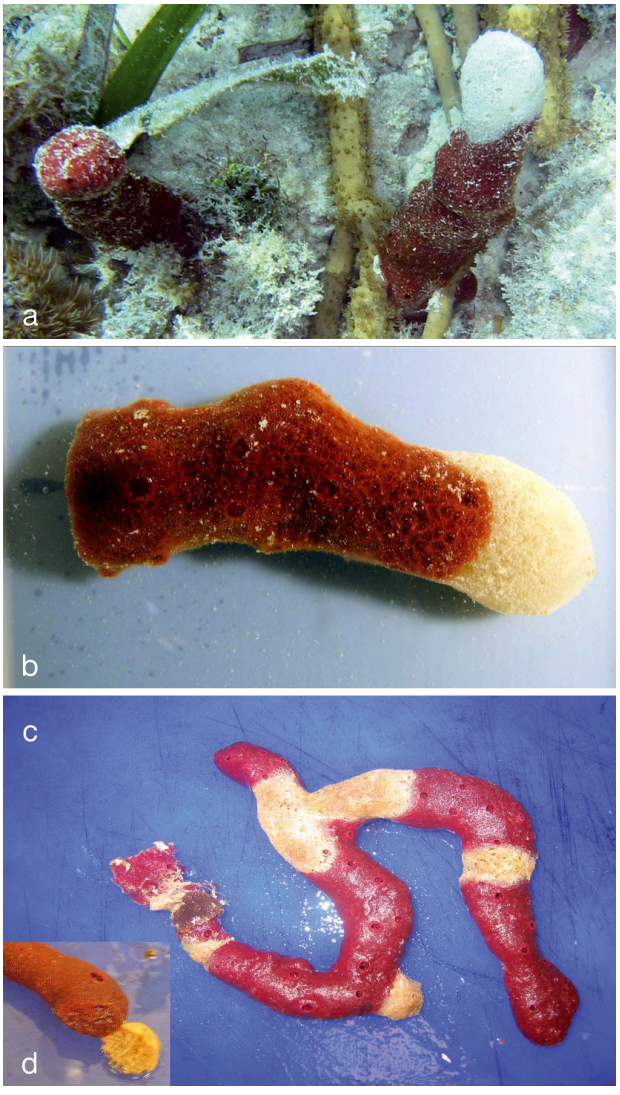

Fig. 1. *Amphimedon compressa*. (a) Underwater and (b–d) laboratory photographs of individuals with sponge white patch disease collected at either (a,b) Conch or (c,d) Dry Rocks Reef, Florida, USA

cific symptoms of disease. The population density of *A. compressa* at this site was 7.6 ± 0.6 sponges m^{-2}. To quantify the impact of disease for a sponge individual, the amount of diseased tissue per sponge was determined using the volume displacement method. The mean tissue volume of diseased *A. compressa* was 59.0 ± 7.4 ml (n = 40). On average, 78.8% (SD = 0.25, SE = 0.04) of the volume of a diseased individual was healthy red tissue and 21.2% (SD = 0.25, SE = 0.04) was bleached.

To obtain more detailed insights into the disease process, red tissues from healthy individuals of *Amphimedon compressa* (n= 2) as well as red and white SWP tissues from diseased ones (n = 3) were inspected by SEM. The red tissue of healthy individuals (Fig. 2a,b) was indistinguishable from the red tissue of SWP individuals (Fig. 2c,d). Round sponge cells of 5–10 µm size were present in the red parts of both healthy and diseased sponges. In stark contrast, the white tissues of SWP individuals revealed obvious signs of destruction, including ample amounts of detritus, exposure of spicules and the presence of only few intact sponge cells (Fig. 2e,f).

TEM studies were conducted on healthy (n = 5) as well as SWP (red and white tissue; n = 9) specimens of *Amphimedon compressa*. Protective barriers of densely compacted collagen, as were observed separating the diseased and healthy tissues in *Ircinia* sponges (Maldonado et al. 2010), were not observed in the present study. However, spongin-boring bacteria were found within the collagen fibers in 8 of 9 diseased individuals investigated and were seen to produce an elaborate canal system (Fig. 3). The spongin-boring bacteria were approximately 0.5 × 1 µm in size and appeared to be spherical or rod-shaped (Fig. 3c). The images shown in Fig. 3 resemble closely the only confirmed sponge pathogen so far that has been identified as the spongin-boring alphaproteobacterium NW4327 affecting *Rhopaloides odorabile* (Webster et al. 2002). Spongin-boring bacteria were also observed in the spongin matrix of healthy *Axinella verrucosa* (Steffens 2003). Interestingly, a close relative of NW4327, termed alphaproteobacterium strain HA007, was isolated from *A. compressa* tissues (99.4% sequence identity over 1375 bp; accession number JQ582943); however, infections of sponges with this isolate did not result in a disease phenotype. Whether the spongin-boring bacteria of *A. compressa* are true pathogens in the sense of the Koch's postulates or whether they are merely secondary opportunistic colonizers that degrade the exposed spongin matrix remains to be determined in future studies. Nonetheless, the correlation between distinctive spongin-boring bacterial morphotypes in bleached tissues of both *R. odorabile* and *A. compressa* and the manifestation of a disease condition in both sponge species is a relevant finding which deserves further attention.

To elucidate the microbial community changes during disease progression, the microbial fingerprints of the red versus white tissue of 3 SWP specimens of *Amphimedon compressa* were assessed by DGGE and compared with the microbial fingerprint of one healthy individual (Fig. 4). Interestingly, the dominant DGGE band (#10) common to all red tissues was absent in all white tissues. The bacterial clone obtained from this DGGE band was most closely

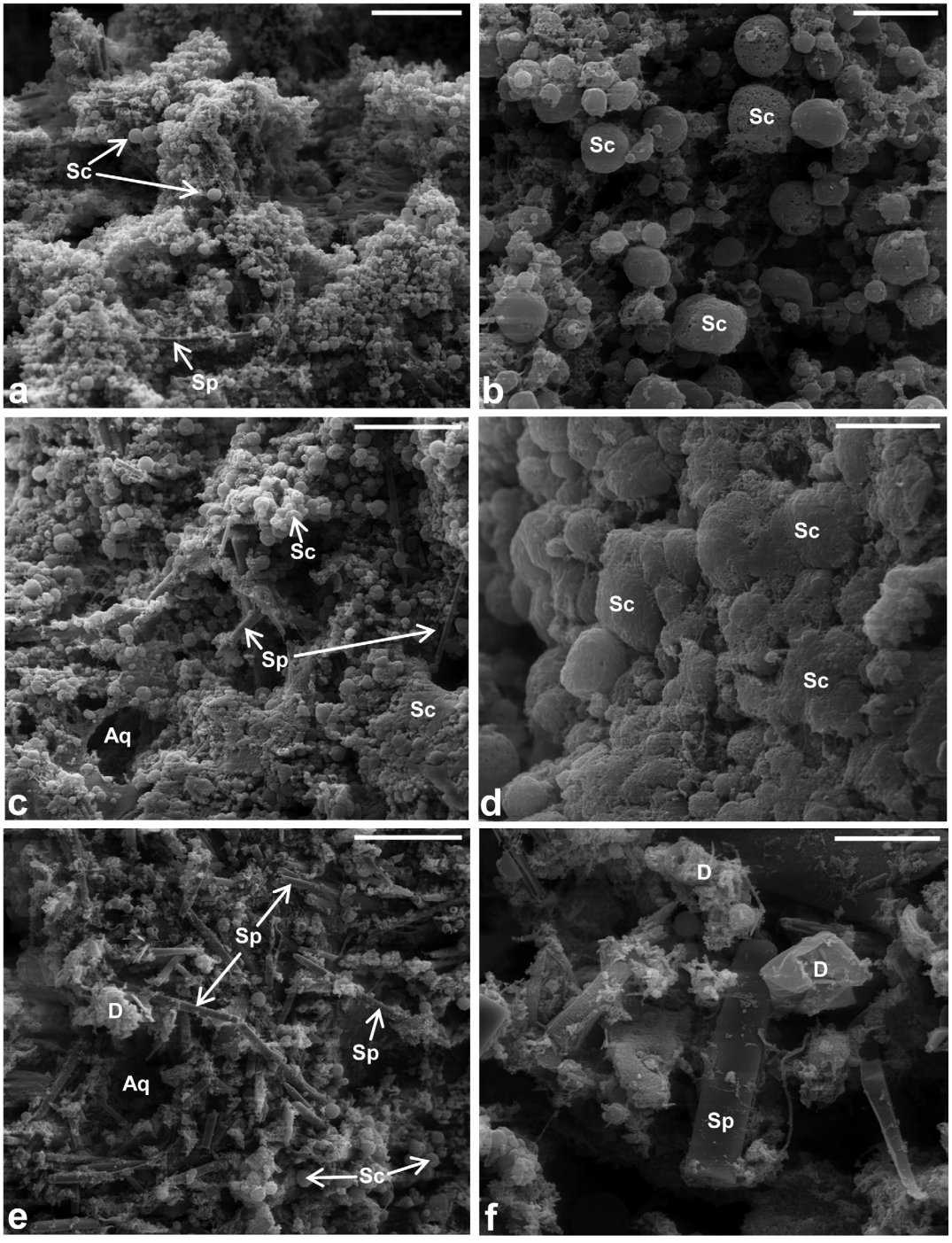

Fig. 2. *Amphimedon compressa*. Scanning electron micrographs of a (a,b) healthy individual and the (c,d) red and (e,f) white tissues of a sponge white patch (SWP) individual. Scale bars = (a,c,e) 50 µm and (b,d,f) 10 µm. Aq: aquiferous canal; D: detritus; Sc: sponge cells; Sp: spicules

related (91% sequence identity) to a 16S rRNA gene sequence derived from another sponge, *Crambe crambe* (Table 1). Six more sequences from healthy *A. compressa* sponges were obtained by DGGE that belonged either to *C. crambe*-associated clone transformant6 (GU799617) or to *C. crambe*-associated clone transformant10 (GU799622) with similarly low sequence identity values (data not shown). In contrast, the DGGE bands from the white *A. compressa* tissues were most closely related to environmental

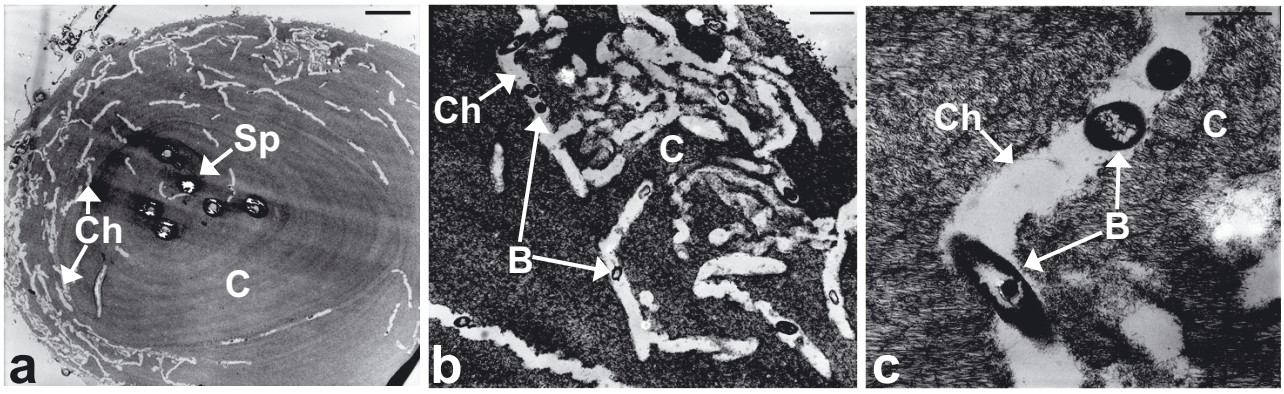

Fig. 3. *Amphimedon compressa*. Transmission electron micrographs of diseased tissues of a diseased individual. B: spongin-boring bacteria; C: sponge collagen; Ch: channels; Sp: spicules. Scale bars = (a) 5 µm, (b) 1 µm and (c) 0.5 µm

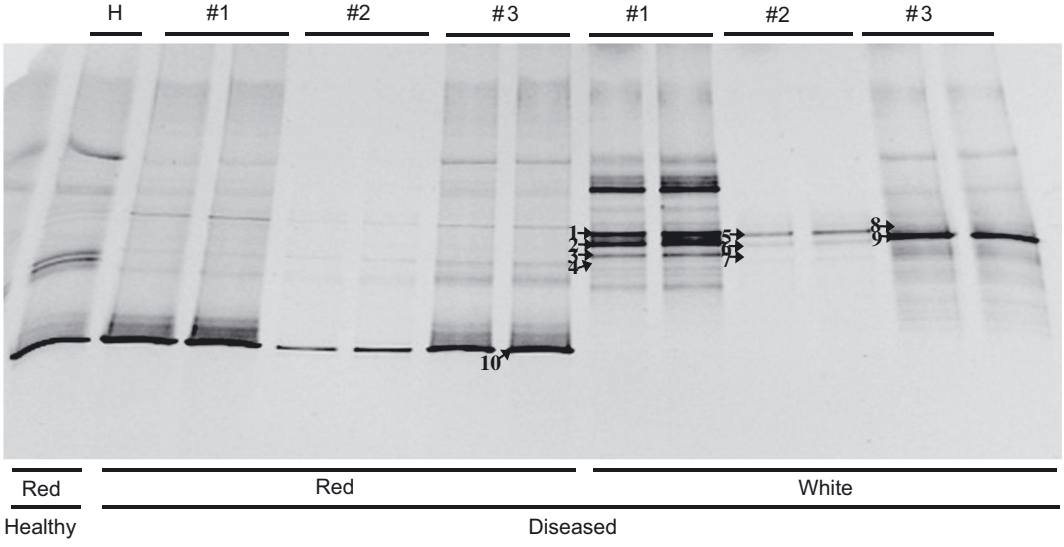

Fig. 4. *Amphimedon compressa*. Denaturing gradient gel electrophoresis fingerprinting of a healthy (H) individual and of the red (R) and white (W) tissues of diseased individuals (#1, #2 and #3). Arrows point towards the excised bands (1–10; see Table 1)

sequences (polluted sands, microbial flocs and engineered microfiltration systems), to a 16S rRNA gene sequence derived from a heat stressed sponge, or to phylotypes implicated in coral diseases (white plague-like syndrome and black band disease). Whether sponges act as reservoirs for coral-disease associated bacteria, as has been proposed previously (Ein-Gil et al. 2009, Negandhi et al. 2010), or whether they are merely colonized by opportunistic bacteria capable of utilizing decaying material remains to be investigated. Indeed, many marine pathogens can be found in the environment, so their recovery from sponge tissues is not unexpected.

The shift from the normal host-associated microbiota towards a different mix of environmental phylotypes has been previously noted in diseases of sponges (Webster et al. 2008, López-Legentil et al. 2010, Angermeier et al. 2011) and corals (i.e. Bourne et al. 2008). Indeed, there has been a recent conceptual change, moving away from focusing on microbial pathogens as disease-causing agents to recognizing the relevance of intrinsic microbial community shifts prior to or during disease progression (Webster et al. 2008, Luter et al. 2010, Angermeier et al. 2011). Even though there is experimental evidence for the presence of bacteria implicated in sponge and coral disease in SWP-afflicted *Amphimedon compressa*, tissue transplantation trials failed to provoke a SWP phenotype. Therefore, the cause of SWP in *A. compressa* cannot be unambigously identified. More detailed experimental time series as well as long-term monitoring studies, taking into account also

Table 1. 16S rRNA gene sequence analysis of the DGGE bands shown in Fig. 4

Tissue	Band	Accession number	Closest sequence match in GenBank	Similarity (%)	Length (bp)	Taxonomic affiliation
Diseased: white	1	HQ659567	Clone SGUS1003 associated with aquarium-kept *Montastraea faveolata* (FJ202771)	99	583/586	*Bacteria*
	2	HQ659568	Clone 33aA1 associated with *Rhopaloeides odorabile* exposed to 33°C (EU183945)	99	439/441	*Bacteria*
	3	HQ659569	*Flavobacteriaceae* isolate DGGE gel band S12 obtained from hydrocarbon and oil spill polluted sand (EU375150)	89	474/531	*Flavobacteriaceae*
	4	HQ659570	Alphaproteobacterium clone 4-7-2 associated with white plague-like syndrome affected *Montastraea annularis* (AF544940)	97	424/433	*Alphaproteobacteria*
	5	HQ659571	Alphaproteobacterium clone RB_18f associated with black band-diseased *Siderastrea siderea* from the Bahamas (EF123421)	98	553/560	*Alphaproteobacteria*
	7	HQ659572	Microfiltration system associated bacterium clone MF-Oct-95 (HQ225056)	96	546/565	*Bacteria*
	8	HQ659573	*Montastrea faveolata* associated *Clostridiaceae* bacterium clone MD3.13 (FJ425601)	96	540/559	*Clostridiaceae*
	9	HQ659574	Clone BBD-Dec07-1BB-2 associated with the black band disease consortium of *Favia* sp. (GQ215183)	99	561/564	*Bacteria*
Diseased: red	10	HQ659575	Sponge *Crambe crambe* associated clone transformant10 (GU799622)	91	538/589	*Bacteria*

environmental and anthropogenic influences, will be required to understand the underlying causes of SWP in *A. compressa* and other sponge and coral populations. Future areas of interest (see Webster & Taylor 2011 for a recent review) will be to identify possible correlations between disease and environmental factors (i.e. temperature, sedimentation, pollution and physical damage by storms), investigate the role of viruses and fungi as causative agents, and localize bacteria within tissues by more specific methods, such as fluorescence *in situ* hybridisation.

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