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Ulcerated yellow spot syndrome: implications of aquaculture-related pathogens associated with soft coral *Sarcophyton ehrenbergi* tissue lesions

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ABSTRACT: We introduce a new marine syndrome called ulcerated yellow spot, affecting the soft coral *Sarcophyton ehrenbergi*. To identify bacteria associated with tissue lesions, tissue and mucus samples were taken during a 2009 Indo-Pacific research expedition near the Wakatobi Island chain, Indonesia. Polymerase chain reaction targeting the 16S rDNA gene indicated associations with the known fish-disease-causing bacterium *Photobacterium damselae*, as well as multiple *Vibrio* species. Results indicate a shift toward decreasing diversity of bacteria in lesioned samples. *Photobacterium damselae* ssp. *piscicida*, formerly known as *Pasteurella piscicida*, is known as the causative agent of fish pasteurellosis and in this study, was isolated solely in lesioned tissues. Globally, fish pasteurellosis is one of the most damaging fish diseases in marine aquaculture. *Vibrio alginolyticus*, a putative pathogen associated with yellow band disease in scleractinian coral, was also isolated from lesioned tissues. Lesions appear to be inflicting damage on symbiotic zooxanthellae (*Symbiodinium* sp.), measurable by decreases in mitotic index, cell density and photosynthetic efficiency. Mitotic index of zooxanthellae within infected tissue samples was decreased by ~80%, while zooxanthellae densities were decreased by ~40% in lesioned tissue samples compared with healthy coral. These results provide evidence for the presence of known aquaculture pathogens in lesioned soft coral and may be a concern with respect to cross-species epizootics in the tropics.

KEY WORDS: *Photobacterium damselae* · Indonesia · *Vibrio* · Zooxanthellae · 16S rDNA · Lesion

INTRODUCTION

The presence of pathogenic bacteria is inherent to any natural system. However, incidences of bacterial infections found in marine organisms have steadily increased over the past 20 yr (Goreau et al. 1998, Ben-Haim et al. 2003, Green & Bruckner 2000, Cervino et al. 2001, Rosenberg et al. 2007). This trend has been reported in finfish (Pulkkinen et al. 2010), crustaceans in aquaculture (Chiu et al. 2007, Rebouças et al. 2011) and various types of coral (Kushmaro et al. 2001, Smith et al. 1996, Goreau et al. 1998, Sutherland et al. 2004, Cervino et al. 2008). Conditions that may have influenced increases in waterborne pathogens may include increased rates of human population growth and development in sensi-

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tive coastal areas (Dorfman & Rosselot 2011) and increased aquaculture activities along shorelines (Naylor et al. 2000). As the disease resistance of coral is thought to decrease with changing environmental conditions (Harvell et al. 1999) and global aquaculture facilities are concurrently increasing in number, the potential for epizootics and novel emerging diseases increases (Garren et al. 2008, Garren et al. 2009).

As aquaculture fish farming is based on the concept of raising monoculture crops at high density, the potential for pathogenic bacteria to exploit these organisms is high. Bacterial infection is the main cause of disease-induced death of fish raised in aquaculture (Johansen et al. 2011). For example, since 1969 the bacterium *Photobacterium damselae* has been consistently associated with aquaculture diseases in Japan, causing infections in yellowtail *Seriola quinqueradiata* (Kusuda & Yamaoka 1972). However, since the 1990s, this pathogen has spread, causing severe economic losses in aquaculture stocks in numerous countries, including France, Italy, Spain, Greece, Turkey, Portugal, Malta and, recently, Malaysia, e.g. La Mesa et al. (2008).

The growth of the aquaculture industry has brought concern about the potential effects of aquaculture pathogens on surrounding populations as the design of many of these farms is open and allows for transmission to the neighbouring environment. However, the implications of aquaculture pathogens on surrounding natural populations has been difficult to classify as the relationships between host, pathogen and the environment are complex and may be influenced by a myriad of factors (Hedrick 1998).

China, India and Indonesia represent ~70% of the world aquaculture market as of 2007, with 60, 6 and 3% of the market respectively (FAO 2007), while harboring nearly 45% of the world's coastal coral reefs (Spalding et al. 2001). The close proximity of coastal aquaculture facilities to coral reef habitats in these regions, with the influences of stock escapement and effluent discharge, amplifies the probability of coral interacting with microbial organisms, both pathogenic and autochthonous species, potentially resulting in coral mucus community shifts, infection and mortality.

As demonstrated by Garren et al. (2009), corals transplanted adjacent to an aquaculture facility in Bolinao, Philippines, and exposed to high concentrations of effluent experienced a shift in microbial populations, resulting in increases in bacterial groups associated with human and coral pathogens. Prior to the development of the aquaculture facility, Garren et al. (2009) reported a native population of corals in this location that has since died. That study also found that after 21 d, the transplanted coral experienced yet another change in microbial communities back to profiles more similar to those seen prior to their exposure to high effluent from the facility. Despite the finding of an initial shift to increased pathogens, the short duration of the study precluded observing causation of coral infection and the development of disease symptoms. However, it did show that pathogenic bacteria derived from aquaculture effluent have the capacity to interact with coral mucus and were a possible cause of death of the prior native population of coral.

Here we present a new coral syndrome called ulcerated yellow spot (UYS) affecting *Sarcophyton ehrenbergi*, a soft coral (common name 'leather coral'). We choose to use the term 'syndrome' or 'disease-like syndrome' to describe the complex of clinical symptoms (Dorland 1982) that we have identified with this coral, as the term 'syndrome' is less readily associated with causation than the term 'disease' and we have not performed any definitive causality experiments. Although diseases of scleractinian corals are becoming more common (Rosenberg & Loya 2004), infections on soft corals are rare (Williams et al. 2011). For example, a recent assessment of the Palmyra Atoll in the Central Pacific found disease in soft corals, compared to stony corals, to be the least prevalent (<0.03% of all disease found) in all reefs surveyed (Williams et al. 2011). This study presents a preliminary analysis of various groups of marine bacteria found on the surface of these soft corals, including healthy tissues and areas displaying yellow lesions, using 16S rDNA gene sequencing for species identification. We also present evidence of impaired zooxanthellae function within lesioned tissues indicated by reduced photosynthesis and decreased densities and mitotic indices. Finally, we discuss the prevalence of UYS throughout the study site, the similarities of bacteria found within lesions to those known to cause diseases in aquaculture species, and implications for the future of these corals and coral reefs.

MATERIALS AND METHODS

UYS was observed in the Wakatobi archipelago of Indonesia (5° 18′ 34.47″ S, 123° 35′ 08.31″ E; Fig. 1). To assess the frequency of these lesions on *Sarcophyton ehrenbergi*, lesioned versus healthy corals were counted along transects (n = 3 per site) at 8

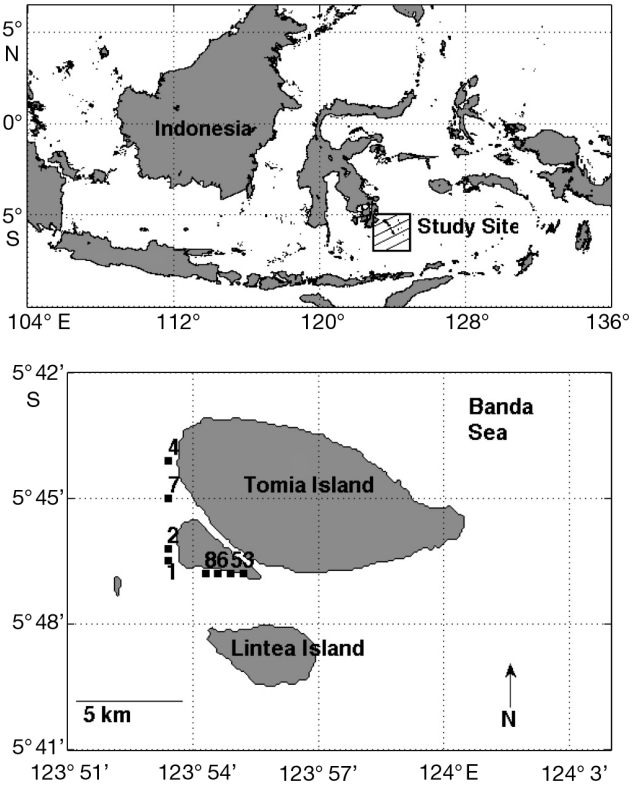

Fig. 1. Study sites, located on the eastern side of Tomia and Onemobaa Islands in the southeast of Indonesia, indicated by the hatched area. Disease transects were carried out at each of the 8 sites: (1) House Reef #1, (2) House Reef #2, (3) Barracuda, (4) Roma, (5) Zoo, (6) Conchita, (7) Table Coral City and (8) Turkey Beach

study sites. Study sites were chosen based upon the emergence of UYS at sites visited during 2009 where the disease was absent in 2004. The names of each site surveyed and their associated site numbers in the island chain were: (1) House Reef #1, (2) House Reef #2, (3) Barracuda, (4) Roma, (5) Zoo, (6) Conchita, (7) Table Coral City and (8) Turkey Beach (Fig. 1). Transect lines (50 × 1 m transects), ~3 m above each reef-flat surface, were established and corals were counted and photographed (Fig. 2) to help determine the frequency of soft coral colonies with UYS lesions versus those coral colonies not infected. Transects were begun at the shore-dive entry point and were continued at increasing depth and distance from the shore (final depth = 18 m). Colonies were photographed using an Underwater Nikon RS (SLR system). Two-way ANOVA was performed with transect site and coral health (lesioned versus healthy regions) as factors and abundance as a response variable (R v. 2.10.1; The R Foundation for Statistical Computing).

Pulse amplitude modulated fluorometry

Along each transect line, measurements of photosynthetic efficiency were taken of lesioned and healthy tissues of every *Sarcophyton ehrenbergi* encountered using a diving pulse amplitude modulated (PAM) fluorometer (Walz). Healthy and lesioned tissues were probed to obtain measurements of zooxanthellae overall quantum yield of photochemical energy conversion (i.e. dF/F_m') as a function of symbiont photosynthetic efficiency. Relative rate of electron transport (ETR) was also measured to ensure that dF/F_m' (yield) readings were indicative of changes in photosynthetic incompetence, rather than fluctuations of environmental parameters. Measurements were taken at each site and transect where healthy versus lesioned coral data were collected. A one-way ANOVA was used to determine the differences in fluorescent yield between the 2 conditions of health (R v. 2.10.1). We further analyzed the relationship between other parameters measured by the Diving PAM — photosynthetically active radiation, ETR and yield — as well as depth, using multiple logistic regression, in order to determine which of these parameters were best used to predict lesions. Variance inflation factors were used to simplify the model with a cut-off value of 2.5. Data exploration was carried out using scatterplots of the independent variables and R^2 coefficients.

Sample collection of *Sarcophyton ehrenbergi*

Coral tissue samples were collected for zooxanthellae analysis using SCUBA. For each targeted colony, one 2.5 cm^2 tissue sample was taken using stainless steel scissors. One sample was taken within the lesion of the affected coral while the other sample was taken from a separate healthy coral. At each survey site 6 corals were sampled, 3 lesioned and 3 healthy. Individual tissue samples were placed in 100 ml polyethylene bottles in filtered seawater (FSW). Samples were kept on ice in coolers during transit back to the laboratory on shore.

Mitotic index

In the laboratory, tissue was removed from all specimens using a Water Pik following the methods of Johannes & Wiebe (1970). The liquid extract containing tissue and zooxanthellae was homogenized and centrifuged at 10 000 × *g* for 5 min in order to collect

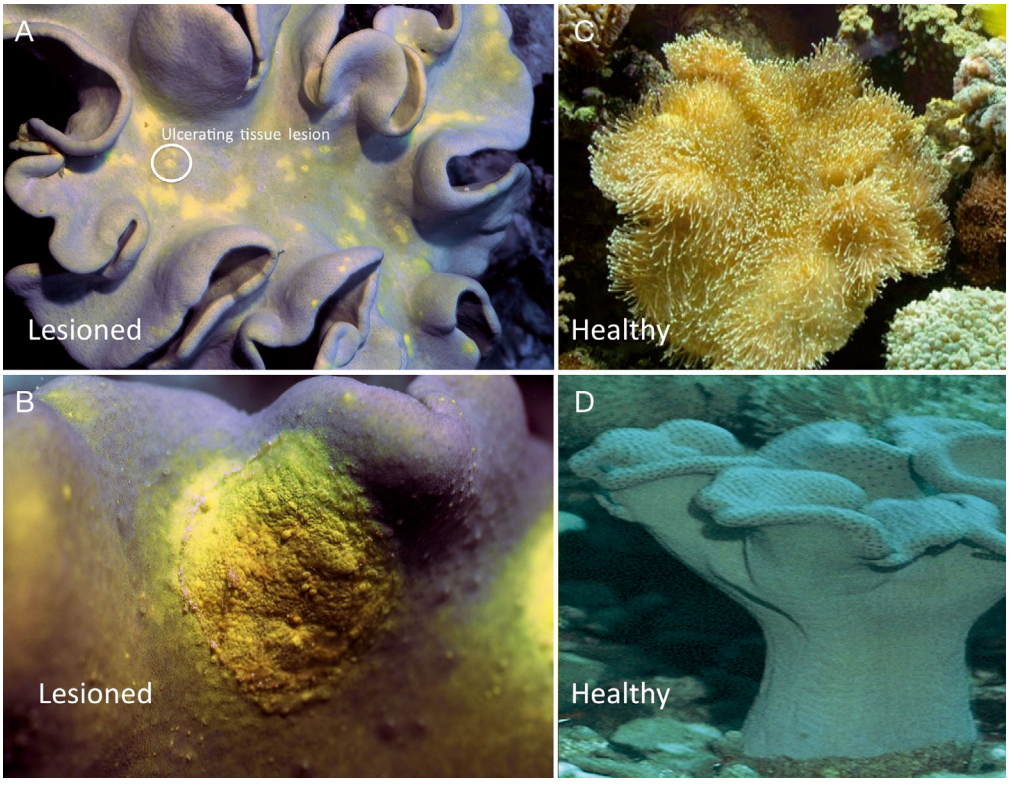

Fig. 2. Lesioned and healthy *Sarcophyton ehrenbergi*. (A) *S. ehrenbergi* displaying areas of yellow lesioned tissue. (B) Magnified image of ulcerated lesion. A healthy *S. ehrenbergi* is not included here but looks like the coral shown in (A) minus the yellow tissue. (C,D) Examples of a healthy *S. ehrenbergi*

zooxanthellae. Supernatant was discarded, and the pellet was re-suspended in 1 ml of 10% gluteraldehyde, 15% PBS, FSW solution and stored at 20°C. Zooxanthellae density and mitotic indices (MI%) were determined from each 2.5 cm tissue sample. These were determined by direct examination under a Zeiss ApoTome Fluorescence microscope at ×20 to ×100 using a Neubauer hemocytometer following Wilkerson et al. (1988). Welch 2-sample *t*-tests were used to determine differences in mitotic and zooxanthellae cell abundances between the 2 conditions of health (R v. 2.10.1).

Sample collection for bacterial isolation

Microbial analysis consisted of 6–10 ml sterile syringe samples drawn from the surface layers of lesioned and healthy *Sarcophyton ehrenbergi* from 3 dive locations using SCUBA. Sampling sites were determined based on accessibility and time constraints, as each site had high levels of lesion occurrence (Fig. 2). At each of the 3 dive sites, one ulcerated coral was chosen for surface mucus samples as well as one healthy coral, thereby totalling 6 corals sampled for bacterial analysis. Although we realize this is a fairly low replication number, constraints regarding boat availability, time and supplies led to our sample sizes.

To minimize between-sample contaminations, each sample was taken by a diver wearing gloves, using a sterile, labelled syringe, and stored in individual plastic bags for transport to the surface. Samples were taken within the ulcerated lesion and on healthy tissue. Syringe samples were then transferred to the surface and immediately inoculated onto Glycerol Artificial Sea Water (GASW) (Smith & Hayasaka 1982) slants.

Due to restricted availability of a laboratory in Indonesia, samples were re-inoculated upon return to the USA in liquid GASW medium for 16–24 h at 26°C (the average water temperature during all sampling events). Each original sample (at concentration C) was then serially diluted in 1 ml of liquid media in 6–15 ml falcon tubes to a final concentration of $10^{-6}C$. The entire sample (1 ml) was then plated on GASW plates for dilutions of $10^{-6}C$ and $10^{-5}C$ and incubated for 16 to 24 h at 26°C.

Post incubation, single colonies were isolated via colony morphology, including size, shape and color. Each individual colony was subsequently circled (i.e. on the back of the Petri dish), labelled and re-plated on GASW plates using a sterile toothpick. Overall, 135 pure cultures were isolated, 74 originating from healthy tissue samples and 65 originating from lesioned coral samples.

PCR of bacterial 16S rDNA gene

Bacterial isolates taken from each isolated colony were amplified using 16S rDNA. The forward bacterial primer Bact-8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and the reverse bacterial primer Bact-1512R (5′-GGT TAC CTT GTT ACG ACT T-3′) were used (Weisburg et al. 1991). Amplification mixtures contained 30 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM (each) deoxynucleoside triphosphates, 150 pmol of each primer and 5 U *Taq* polymerase (Promega) in a final volume of 100 µl. Sterile toothpicks were used to transfer a small amount of each isolate to the PCR reaction tubes. A control strain of *Vibrio alginolyticus* (R-370) was used, as well as negative controls containing no DNA templates to ensure non-target sources of bacteria were not amplified. PCR was conducted with an Eppendorf Mastercycler Personal. PCR amplification consisted of 2 min denaturation at 94°C, followed by 35 cycles of 1 min denaturation at 94°C, a 1 min annealing at 40°C, 1 min 30 s extension at 72°C with a final 5 min extension at 72°C after completion of the last cycle. Finally, 5 µl of the PCR reaction was visualized on 1% agarose containing SYBR® Safe DNA gel stain (Invitrogen), with a UV light.

DNA sequencing

DNA was sequenced on a Beckman-Coulter DNA automated sequencer (Applied Biosystems). Sequence chromatograms of forward and reverse sequences were edited and assembled using Sequencher 4.8 (Gene Codes). The sequences were then aligned using BioEdit Sequence Alignment Editor version 7.0.9.0 (Hall 1999) and ClustalX (Thompson et al. 1997). A maximum likelihood tree was developed using MEGA5 and edited in Fig Tree v1.3.1. The tree was rooted using *Enterovibrio coralii*. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was <100 or less than one-fourth of the total number of sites, the maximum parsimony method was used; otherwise the BIONJ (Biological Neighbor Joining) method with an MCL (Markov Linkage Clustering) distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 45 nucleotide sequences. Codon positions included were 1st, 2nd and 3rd noncoding. All positions containing gaps and missing data were eliminated. There were a total of 628 positions in the final data set. Evolutionary analyses were conducted in MEGA5.

RESULTS

Transect data show a significantly higher frequency of lesioned *Sarcophyton ehrenbergi* compared with healthy coral colonies at all 8 sites except

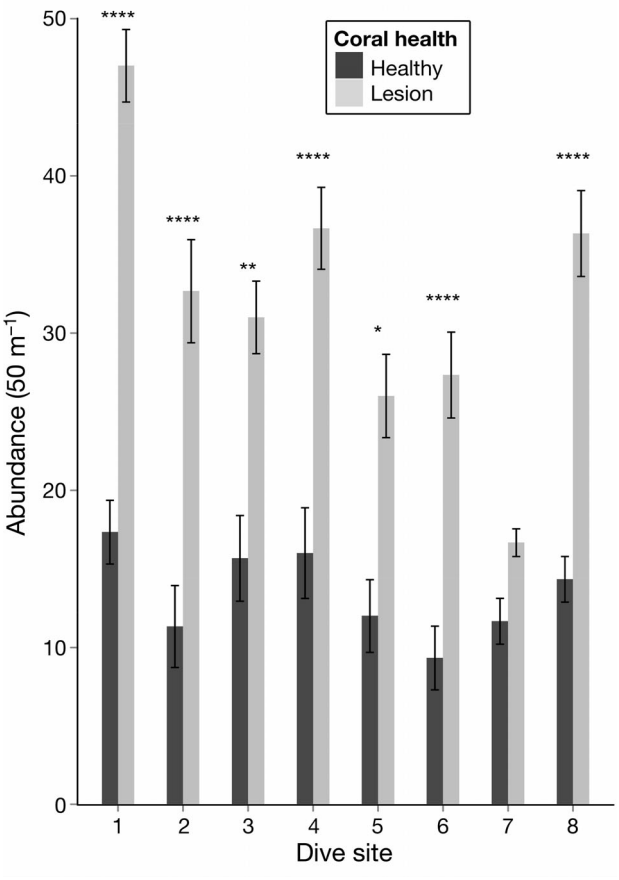

Fig. 3. Mean (±SE) abundance of healthy and lesioned coral along 50 m transects at individual sites. Lesioned coral were significantly more prevalent at all sites except Site 7. Asterisks represent a significant difference between lesioned and healthy coral at a particular site (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

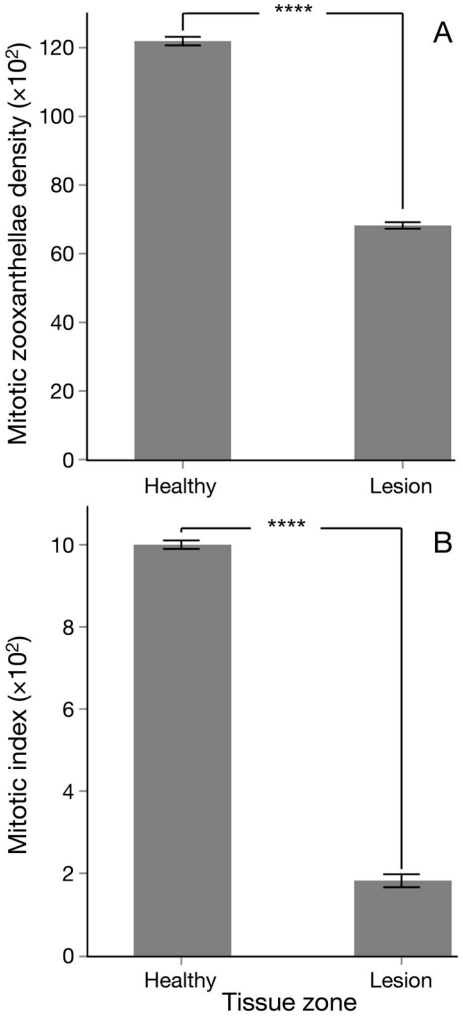

Fig. 4. (A) Mean (±SE) zooxanthellae density and (B) zooxanthellae mitotic index (MI%) for healthy and lesioned tissue samples taken from individual infected *Sarcophyton* sp. colonies. The means were calculated using all sites. Significant differences are reported with asterisks (****$p < 0.0001$)

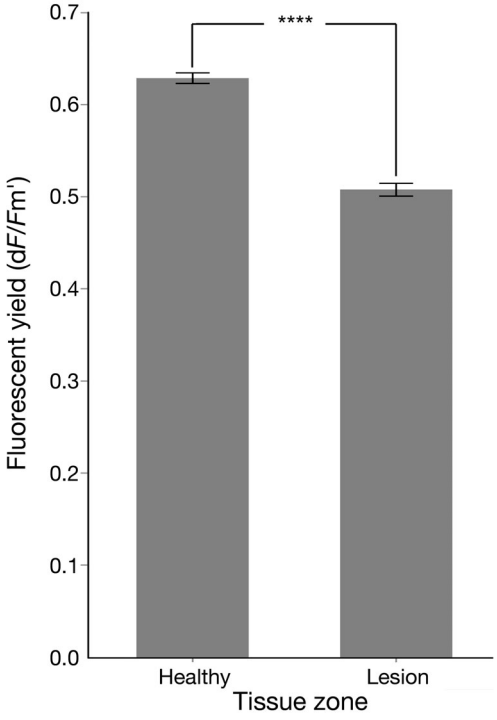

Fig. 5. Mean (±SE) fluorescent yield of healthy versus lesioned corals along 50 m transects recorded at all sites (n = 666 healthy, n = 736 lesioned). Significant differences are reported with asterisks (****$p < 0.0001$)

for Site 7. However, at this site the mean abundance of lesioned coral was still greater than that of healthy individuals (Fig. 3). The mean abundance of lesioned *S. ehrenbergi* was more than double that of healthy *S. ehrenbergi* at nearly all sites (1, 2, 4, 6 and 8). At all 8 sites, the size of yellow lesions affecting *S. ehrenbergi* was similar, with the average lesion size ~1 to 2 cm. Lesions on individual coral did not seem to be spatially or temporally correlated. However, coral with lesions seemed to be closer together, rather than haphazardly distributed throughout the reef.

Mean density of zooxanthellae in healthy tissues was significantly ($p < 0.0001$) different from cell densities found in lesioned tissue (Fig. 4A), with densities in healthy tissue ~50% greater than those of lesioned tissues. MI% was also significantly ($p < 0.0001$) different in healthy tissues compared with lesioned tissues, with a 5-fold increase in mitotic cells from lesioned to healthy tissue (Fig. 4B). During MI% counts, it was also noted that zooxanthellae from lesioned tissue were in poorer physiological condition than those from healthy tissue.

Results of PAM analysis also demonstrated a disruption of zooxanthellae photosynthetic efficiency, with mean fluorescent yield (dF/F_m') of lesioned tissue significantly lower ($p < 0.0001$) than that of healthy tissue (Fig. 5). ETRs were also significantly lower ($p < 0.0001$) in lesioned versus healthy tissue. Multiple logistic regression indicated yield to be significantly different between healthy and lesioned tissue. Predicted values from this model indicate that a yield of 0.3 will provide an ~95% probability of indicating non-healthy tissue. Regression analysis also indicated that depth and ETR add significantly to the determination of lesioned tissue; however, odds ratios for these variables were 1.289 and 1.031, respectively, and therefore less predictive than yield. ETRs <10 indicated an 80% chance of non-healthy tissue. Shallower depths increased the probability of finding non-healthy tissue; however, within the range of depths sampled, a high degree of variability existed

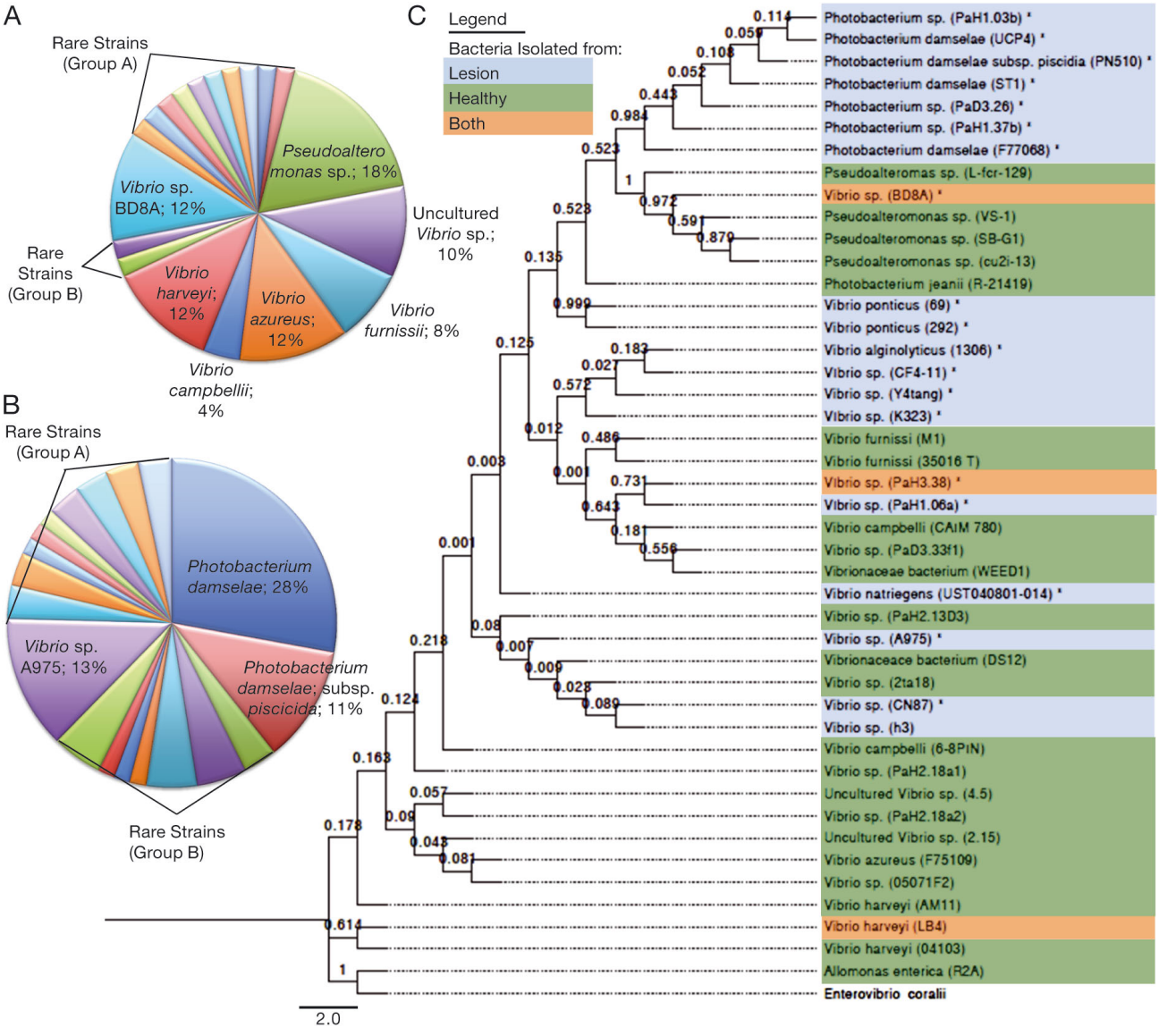

Fig. 6. (A) Bacterial strains isolated from healthy *Sarcophyton ehrenbergi* tissue samples. Strains with a small number of replicates were designated as 'rare' for figure clarity and separated into groups A and B for identification in the figure legend. Isolates in rare group A (all at 2%) are *Vibrio* sp. (H3), *Vibrio* sp. (PaD3.33f1), *Vibrio* sp. (PaH2.13D3), *Vibrio* sp. (PaH3.38), *Vibrio* sp. (PaH2.18a1), *Vibrio* sp. (PaH2.18a2), Vibrionaceae bacterium (DS12), Vibrionaceae bacterium (WEED1), *Photobacterium jeanii*, and *Allomonas enterica*. Isolates from rare group B (both at 2%) are *Vibrio* sp. (2ta18) and *Vibrio* sp. (05071F2). (B) Bacterial strains isolated from lesioned *S. ehrenbergi* tissue samples. Isolates in rare group A, with their percentages, are *Vibrio* sp. (BD8A) (3%), *Vibrio* sp. (CF4-11) (3%), *Vibrio* sp. (CN87) (2%), *Vibrio* sp. (K323) (2%), *Vibrio* sp. (PaH1.06a) (2%), *Vibrio* sp. (PaH1.24) (3%), *Vibrio* sp. (PaH3.38) (3%), *Vibrio* sp. PaH3.39 (3%), and *Vibrio* sp. (Y4tang) (3%). Isolates in rare group B are *Vibrio ponticus* (5%), *Vibrio natrigens* (UST040801-014) (2%), *Vibrio alginolyticus* (1306) (2%), *Vibrio harveyi* (LB4) (2%), *Photobacterium* sp. (PaH1.37b) (5%), *Photobacterium* sp. (PaH1.03b) (5%), and *Photobacterium* sp. (PaD3.26) (3%). (C) The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (–3369.9) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. *Strains isolated in lesion tissue sample

with respect to lesioned tissue occurrence. We therefore believe that depth does not play a large role in terms of lesion frequency.

As described earlier, we isolated 135 bacterial colonies. However, only 113 isolates could be sequenced successfully (51 from healthy and 62 from lesioned coral tissues). Unsuccessful sequences were likely due to poor quality DNA amplification during PCR reactions. Results of 16S rDNA sequencing revealed *Vibrio* (74%) and *Pseudoalteromonas* (18%)

Table 1. Complete list of bacterial strains isolated from healthy and lesioned *Sarcophyton ehrenbergi* tissue samples. n: number of each strain isolated in healthy tissue; identity: percentage identity to closest-match bacterial representative in GenBank database. Asterisks (*) indicate strains isolated in healthy and lesioned tissue samples

Name	n	GenBank accession number	Identity (%)
Healthy samples			
Allomonas enterica	1	HQ908698.1	99
Photobacterium jeanii (R-21419)	1	GU065212.1	99
Pseudoalteromonas sp. (cu2i-13)	3	JN594612.1	100
Pseudoalteromonas sp. (SB-G1)	2	AB457053.1	98
Pseudoalteromonas sp. (VS-1)	4	FJ497708.1	99
Uncultured *Vibrio* sp. (2.15)	1	AM930481.1	99
Uncultured *Vibrio* sp. (4.5)	4	AM930497.1	98
Vibrio furnissii (35016 T)	1	X76336.1	99
Vibrio azureus (F75109)	6	HQ908698	99
Vibrio campbelli (CAIM 780)	1	FM204852.1	99
V. campbelli (6-8PIN)	1	GQ203112.1	99
Vibrio furnissi (M1)	3	EU204961.1	98
Vibrio harveyi (AM11)	1	AB512470.1	99
V. harveyi (04103)	1	AM422802.1	99
V. harveyi (LB4)*	4	FJ952784.1	99
Vibrio sp. (05071F2)	1	HQ449441.1	99
Vibrio sp. (2ta18)	1	FJ952784.1	98
Vibrio sp. (BD8A)*	6	EU372939.1	95
Vibrio sp. (h3)	1	EF187010.1	98
Vibrio sp. (PaD3.33f1)	1	GQ406671.1	99
Vibrio sp. (PaH2.13D3)	1	GQ406760.1	99
Vibrio sp. (PaH2.18a1)	1	GQ391955.1	99
Vibrio sp. (PaH2.18a2)	1	GQ391956.1	99
Vibrio sp. (PaH3.38)*	1	GQ406800.1	99
Vibrionaceae bacterium (DS12)	1	EF584044.1	98
Vibrionaceae bacterium (WEED1)	1	EF584103.1	99
Lesioned samples			
Photobacterium damselae (DFIL2.4)	1	FR873782	100
P. damselae (F77068)	1	JF281756	100
P. damselae (ST1)	14	GU228793.1	99
P. damselae (UCP4)	1	DQ530294.1	99
P. damselae ssp. *piscidia* (PN510)	6	AY1478601	99
P. damselae ssp. *piscidia* (SWZX5)	1	EF643517.1	99
Photobacterium sp. (PaD3.26)	2	GQ392013.1	98
Photobacterium sp. (PaH1.03b)	3	GQ406683	99
Photobacterium sp. (PaH1.37b)	3	GQ406722.1	99
Vibrio alginolyticus (1306)	1	GU726845.1	99
Vibrio harveyi (LB4)*	1	DQ146935.1	99
Vibrio natriegens (UST040801-014)	1	EU834012.1	98
Vibrio ponticus (292)	2	AJ630202.1	98
V. ponticus (69)	1	NR02032.1	97
Vibrio sp. (A975)	8	GU223593.1	99
Vibrio sp. (BD8A)*	2	EU372939.1	95
Vibrio sp. (CF4-11)	2	FJ169995.1	99
Vibrio sp. (CN87)	1	EU413955.1	99
Vibrio sp. (K323)	1	GU223600.1	99
Vibrio sp. (PaH1.06a)	1	GQ406687.1	99
Vibrio sp. (PaH1.24)	2	GQ406711.1	97
Vibrio sp. (PaH3.38)*	2	GQ406800.1	99
Vibrio sp. (PaH3.39)	2	GQ40680.1	99
Vibrio sp. (Y4tang)	2	EF187013.1	99

as the predominant genera represented in cultures isolated from healthy tissue (Table 1, Fig. 6A,C). Single isolates of *Photobacterium jeanii* and *Allomonas enterica* were also present in healthy tissue samples. Other notable bacterial species represented in healthy *Sarcophyton ehrenbergi* tissue include *V. furnissi*, *V. campbelli*, *V. azureus* and *V. harveyi*.

Vibrio spp. were also largely represented (48%) in lesioned tissues; however, most were different strains from those isolated from healthy tissues (Fig. 6B,C, Table 1). Notably, *Vibrio alginolyticus*, a known coral disease-causing bacterium (Cervino et al. 2004, 2008), was present in lesioned samples. Unlike healthy tissue, however, there was a large presence of *Photobacterium* spp. (52%) in lesioned coral tissues. Four separate strains of *Photobacterium damselae* were identified, as well as 2 strains of *P. damselae* ssp. *piscidia*. Three other strains of *Photobacterium* spp. were also identified, but not to the species level. Other notable bacteria identified in lesioned tissues include *V. harveyi*, *V. natriegens* and *V. ponticus*.

Three *Vibrio* strains were isolated in both healthy and lesioned coral tissue samples (Fig. 6C, Table 1). These included *V. harveyi* (LB4), *Vibrio* sp. (BD8A) and *Vibrio* sp. (PaH3.38). Although present in both healthy and lesioned coral tissue samples, *Vibrio* spp. were found in different concentrations. For example, 4 isolates of *V. harveyi* (LB4) were identified in healthy tissues, whereas only one was found in lesioned coral samples. Six isolates of *Vibrio* sp. (BD8A) were isolated in healthy coral tissue, while only 2 were found associated with lesioned coral tissue, and 2 *Vibrio* sp. (PaH3.38) were found with lesioned and one with healthy coral tissue.

DISCUSSION

As reports of coral and marine diseases and syndromes have increased over the past 30 to 40 yr (Harvell et al. 1999,

Porter et al. 2001, Sweatman et al. 2001), it has frequently been argued whether these diseases are 'novel', have always been present but are increasing in incidence and severity, or have recently begun to be noticed more readily as we become more aware of their presence (Ward & Lafferty 2004). Although UYS may have been present on *Sarcophyton ehrenbergi* colonies prior to 2009, its frequency and intensity had not been large enough for documented observation, research and publication. It is also important to note that at these locations our group identified no lesions during the initial 2004 research trip. In 2009 we observed that all sites contained significantly ($p < 0.01$) more *S. ehrenbergi* colonies with lesions than without, except at Site 7 (Fig. 3). Based on sample site locations, this symptom seems to be spread evenly along the east coast of Tomia and Onemobaa Islands, part of the Wakatobi archipelago.

The cellular impairment mechanisms associated with diseases such as yellow band disease (YBD) are unusual when compared with those of other coral stressors. With YBD, the pathogenic strains of bacteria associated with the disease (*Vibrio* spp.) are attacking the symbiotic algae *in vivo* (Cervino et al. 2004, 2008, Roff et al. 2008) instead of resulting in symbiont expulsion, as is common during stress-related coral bleaching (Gates et al. 1992, Strychar et al. 2004). Other disease-causing *Vibrio* spp. have been isolated from Mediterranean and Red Sea corals, and these appear to attack the symbiotic zooxanthellae living in the tissues of gorgonians and reef builders (Ben-Haim et al. 1999). Results from our study indicate the possibility of ulcerated lesions on the surface of the coral that create a microbial mat, which may be decreasing light penetration into the gastroderm where the zooxanthellae reside. This effect is negatively impacting zooxanthellae function via decreases in zooxanthellae MI% (Fig. 4B) and photosynthetic efficiency observed in overall quantum yield of photochemical energy conversion (dF/F_m') (Fig. 5). Zooxanthellae density also decreased significantly (Fig. 4A) from healthy to lesioned coral tissue ($p < 0.0001$), although the mechanism behind this decrease in density is not yet known (i.e. expulsion versus degradation). Further, previous studies on coral diseases have shown causative pathogens to produce extracellular toxins, which negatively impact the symbiont (e.g. Banin et al. 2000). Although we did not directly address the potential production of bacterial toxins, future studies of these isolates may benefit from such an investigation.

Results obtained from sequencing culturable bacteria isolated from lesioned versus healthy coral tissue suggest 2 different states of microbial associations, regardless of low replication rates from tissue sampling. Healthy coral tissue was found to have a greater overall diversity of bacteria compared with lesioned coral tissue (Fig. 6A,B). Previous studies of bacteria associated with diseased and healthy tissue in coral have shown a decrease in bacterial diversity as well as shifts towards *Vibrio* spp. dominance in infected coral during periods of elevated temperatures (e.g. Ritchie 2006). *Vibrio* is a common genus found in coral-associated microbial assemblages during healthy and disease states (Ritchie & Smith 2004, Bourne & Munn 2005, Ritchie 2006). Interpretation of these results is speculative as the methodologies used here may have created competitive exclusion of culturable microbial constituents and therefore may not express the true degree of culturable bacterial diversity between these 2 tissue conditions.

In addition to known species of non-pathogenic *Vibrio*, previous studies have found *Pseudoalteromonas* sp. to be regularly associated with healthy coral (e.g. Rohwer et al. 2001), similar to our results. In the present study, *Vibrio* represented 48% of the total culturable species isolated from lesioned tissues and 74% in healthy tissues. However, the species or strain of *Vibrio* isolated in each tissue type was different, with those isolated in lesioned tissue being more readily associated with pathogenicity and disease.

Multiple *Vibrio* species have been identified as pathogenic to coral, including but not limited to *V. harveyi*, *V. alginolyticus*, *V. shiloii* and *V. coralliilyticus* (Kushmaro et al. 1996, Ben-Haim et al. 2003, Cervino et al. 2004, Luna et al. 2010). As seen in Table 1, one strain of *V. alginolyticus* was identified in our samples from lesioned coral tissues. *Vibrio alginolyticus* has been previously implicated as a member of the putative YBD-causing consortium of bacteria (Cervino et al. 2004, 2008) along with 4 other novel *Vibrio* strains. *V. alginolyticus* isolates have also been isolated from numerous aquaculture sites worldwide, indicating that it may be the most ubiquitous *Vibrio* phenotype found in diseased crustaceans, marine mammals (Buck et al. 1991) and finfish (Colwell & Grimes 1984, Austin et al. 1993).

Along with *Vibrio alginolyticus*, several more notable bacterial species were isolated in our samples, including *V. ponticus*, *Photobacterium damselae* and *P. damselae* ssp. *piscidia*. *V. ponticus* has previously been isolated from diseased and non-diseased conditions (Macián et al. 2004). *Photobacterium* spp. are Gram-negative, halophilic bacteria found in the family *Vibrionacea*, are commonly found in marine seawater and can be isolated from coastal waters and

sediments all over the world (Rubin & Tilton 1975). This pathogen is known to cause systemic infection in aquaculture fish leading to high rates of mortality (Botella et al. 2002, Pujalte et al. 2003). *P. damselae* is associated with 2 different subspecies, ssp. *damselae* (formerly known as *Vibrio damselae*) and ssp. *piscicida* (formerly known as *Pasteurella piscicida*) (Gauthier et al. 1995). Although both subspecies are considered to be pathogenic in marine fish, it has been shown that only ssp. *piscicida* causes infections with high rates of mortality (Botella et al. 2002, Pujalte et al. 2003). *P. damselae* ssp. *piscicida* is the causative agent of pasteurellosis (also known as pseudotuberculosis), known as one of the most significant fish diseases in marine aquaculture (Magariños et al. 1996). Romalde et al. (2002) has shown that this species becomes pathogenic when sea surface temperatures increase above 18–20°C. Below this temperature, however, fish can carry the pathogen as a subclinical infection and may be carriers for extended time periods (Romalde 2002). In our study these strains were associated exclusively with lesioned coral tissue.

Three species of *Vibrio* were found in both healthy and lesioned coral samples: *Vibrio* sp. (PaH3.38), *V. harveyi* (LB4) and *Vibrio* sp. (BD8A). *Vibrio* sp. (PaH3.38) was originally isolated from a healthy *Pseudopterogorgia americana* mucus sample (Vizcaino et al. 2009), while *V. harveyi* (LB4) was isolated from *Montipora aequituberculata* infected with white syndrome (Sussman et al. 2008) and *Vibrio* sp. (BD8A) was isolated from a diseased fish in an aquaculture pond (Wang et al. 2010). With the exception of *Vibrio* sp. (PaH3.38), fewer numbers of the remaining strains were isolated from lesioned versus healthy coral tissue. Although there seems to be a difference in density of these bacteria between healthy and lesioned tissues, isolation of the same bacterial strains, some of which may be associated with disease, may be a result of the beginning of infection, but not yet providing visual evidence of a lesion. For example, a study by Ritchie (2006) indicated a population shift of *Vibrio* species on the surface mucus of corals in healthy versus diseased states (see also Ritchie & Smith 1997, Rohwer et al. 2001).

The goal of the molecular portion of this study was not to identify the causative pathogens of this syndrome, but to preliminarily identify some of the bacteria associated with lesioned and healthy tissues as well as to describe the complex of symptoms associated with these yellow ulcerated lesions. As the methodologies used here (i.e. GASW medium, growth temperature and time, non-degenerate 16S PCR primers, etc.) are highly selective, interpretation of these preliminary results is speculative. Here we also defined a lesion as any functional or morphological abnormality in coral tissues (Thompson 1978). Accordingly, lesions may be caused by many things besides bacterial infection, including but not limited to predation, human diving-related injuries, nutritional deficiencies and exposure to pollutants (Work & Aeby 2006). Because the goal of our study was not to determine the etiology of these lesions, we cannot directly state that bacteria are the cause. However, our results do yield potential links between aquaculture-associated pathogens and a new marine syndrome affecting *Sarcophyton ehrenbergi* in the Wakatobi archipelago of Indonesia, as known aquaculture pathogens were found associated solely with lesioned tissues. Our data also indicate that this syndrome is causing inhibition of symbiotic algal photosynthesis and replication, similar to the effects of known coral diseases (e.g. YBD) (Cervino et al. 2004, 2008).

Although key species of bacteria have been identified in this study, Fuhrman & Campbell (1998) suggest that, in a given marine sample, more than 95% of bacteria cannot be cultured. Considering this, future studies regarding this syndrome should consider metagenomic analysis of lesioned versus healthy mucus samples of *Sarcophyton ehrenbergi* as well as determining background microbial communities via sampling of the surrounding sediment and water column for a comparative analysis. Various metagenomic-sequencing techniques of environmental samples will allow detection of unculturable species, which is likely to be important when attempting to assess the coral microbial microbiome (Wegley et al. 2007, Vega Thurber et al. 2009). It has also been suggested by Dinsdale et al. (2008) that taxonomic evaluation of microbes associated with an environment through 16S rDNA sequencing is less efficient at describing microbiome characteristics than functional profiling of microbes through full genome sequencing.

The links between coral infections, high organic matter concentrations and changing microbial diversity over the past 30 yr appears to be exacerbated by human development, affecting sensitive tropical coastal environments. Finding and isolating identical fish pathogens from both aquaculture farms and the surfaces of corals would be strong evidence that anthropogenic sources of pollution may be affecting corals. From a pathology perspective, future research is needed to isolate identical fish pathogens at aquaculture farms and compare them with isolated pathogens found on corals, including re-infectivity studies

that satisfy Koch's postulates. Results from this study demonstrate the need for rethinking the way humans utilize coastal zones all over the world in order to help control infectious marine diseases and promote a healthier and sustainable coastal environment.

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