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Assessment of the microbial communities associated with white syndrome and brown jelly syndrome in aquarium corals

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Abstract

Bacterial and ciliate assemblages associated with aquarium corals displaying white syndrome (WS) and brown jelly syndrome (BJS) were investigated. Healthy (n = 10) and diseased corals (WS n = 18; BJS n = 3) were analysed for 16S rRNA gene bacterial diversity, total bacterial abundance and vibrio-specific 16S rRNA gene abundance. This was conducted alongside analysis of 18S rRNA gene sequencing targeting ciliates, a group of organisms largely overlooked for their potential as causal agents of coral disease. Despite significant differences between healthy and diseased corals in their 16S rRNA gene bacterial diversity, total bacterial abundance and vibrio-specific rRNA gene abundance, no dominant bacterial ribotypes were found consistently within the diseased samples. In contrast, one ciliate morphotype, named Morph 3 in this study (GenBank Accession Numbers JF831358 for the ciliate isolated from WS and JF831359 for the ciliate isolated from BJS) was observed to burrow into and underneath the coral tissues at the disease lesion in both disease types and contained algal endosymbionts indicative of coral tissue ingestion. This ciliate was observed in larger numbers in BJS compared to WS, giving rise to the characteristic jelly like substance in BJS. Morph 3 varied by only 1 bp over 549 bp from the recently described Morph 1 ciliate (GenBank Accession No. JN626268), which has been shown to be present in field samples of WS and Brown Band Disease (BrB) in the Indo-Pacific. This result indicates a close relationship between these aquarium diseases and those observed in the wild.

Introduction

The aquarium industry is a significant global enterprise worth $200-300 million annually (Wabnitz *et al.* 2003), with an estimated 11-12 million pieces of coral traded annually (Vincent 2006). High mortality rates of corals in aquaria are therefore a major concern (Sweet *et al.* 2011a). While many cases of coral disease in aquaria are non specific and likely due to deteriorating water quality rather than primary pathogen infections (Borneman and Lowrie 2001; Sweet *et al.* 2011a), there are a number of specific diseases with clearly defined signs (reviewed by Sweet *et al.* 2011a). These include brown jelly syndrome (BJS) and Red Slime Algae which have no reported parallels in nature, and white syndrome (WS), which has similar macroscopic signs to the disease of the same name in the wild (Fig 1). As in the wild, WS within aquariums may progress at different rates (reported to be 0.1 cm per day to 10 cm per hr). The coral diseases with similar macroscopic signs but faster rates of lesion progression are variously known as Rapid Tissue Necrosis, Shut Down Reaction, and Stress Related Necrosis (Hormansdorfer *et al.* 2000; Borneman and Lowrie 2001; Luna *et al.* 2007; Luna *et al.* 2010; Sweet *et al.* 2011a). The slower rates of progression observed in WS within aquaria are similar to those reported for WS in the field (Ainsworth *et al.* 2007; Andersen *et al.* 2010; Luna *et al.* 2010; Work and Aeby 2011).

WS is defined as progressive, full-thickness tissue loss from the coral with a sharp demarcation between the apparently healthy tissue and the denuded white skeleton. Willis *et al.* (2004) defined the term WS to encompass any disease with unknown etiology which showed these particular pathological field signs. Although several studies have implicated specific bacterial pathogens to be the cause of WS (Sussman *et al.* 2008; Luna *et al.* 2010), there has been no evidence of a significant population of bacteria at the disease lesion interface or classical signs of bacterial-induced necrosis (Ainsworth *et al.* 2007; Work and Aeby 2011). Recently, a group of organisms known as ciliates have been shown to be present at the disease lesion interface of WS. These ciliates were shown to ingest the coral tissue and it was suggested that they are responsible for the pathology of these diseases (Sweet and Bythell 2012). This ciliate community was also shown to be similar to that of Brown Band Disease (BrB), another common disease found to occur on the Great Barrier Reef (Sweet and Bythell 2012).

BJS in aquarium corals has been strongly linked to a ciliate, commonly referred to as *Helicostoma nonatum* (Hummon 2008), which has only recently been assigned to the same genus as that of two ciliates in WS and BrB, *Philaster* (Sweet *et al.* 2011a). This study therefore describes both the ciliate and bacterial communities of WS and BJS in aquarium corals using culture-independent (18S and 16S rRNA gene) techniques. Diseased samples were taken opportunistically as they arose in the aquariums, and compared to non-diseased samples collected at the same time from the same aquarium.

Methods

Sample collection

Corals displaying signs of WS were collected from three locations; Horniman Museum and Gardens aquarium in London, the Zoological Society of London (ZSL) aquarium and our own aquarium at Newcastle University. These diseased samples

were compared to apparently healthy corals from the same aquarium systems. Healthy samples (n = 10) and diseased samples (n = 18) of six different species (Table 1) were collected from the various aquariums (Fig 1, Table 1). A further three samples of coral exhibiting symptoms of brown jelly syndrome (Fig 1f), were acquired from ZSL and The Deep (Hull, UK) for analysis of the ciliates previously proposed as the causal agent of this disease. Coral samples were photographed before removal from the aquarium then placed in 50 ml falcon tubes with 100% EtOH and stored at -20°C until extraction and further analysis. Samples were centrifuged at 20,000 rpm for 20 min to concentrate the tissue slurry, 1000 µl of which was subsequently used for DNA extraction using QIAGEN DNeasy Blood and Tissue kits (Sweet *et al.* 2011b) with an added step to concentrate the lysate using a vacuum centrifuge for 2 h at 24° C.

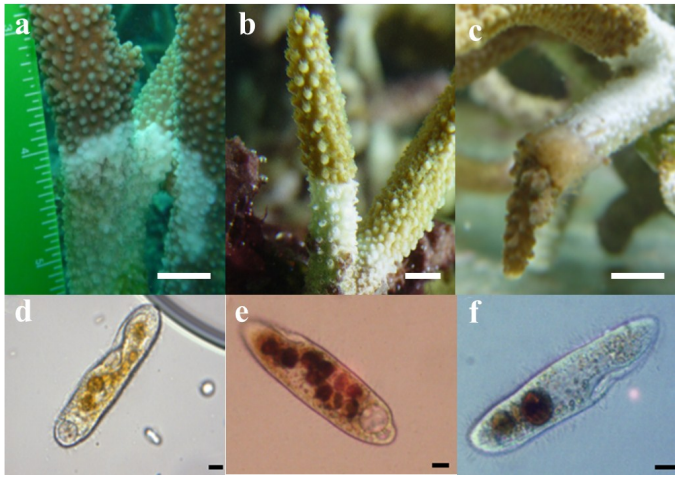

Figure 1. Sub set of samples exhibiting white syndrome (WS) and the dominant ciliates associated with the lesion interface; (a) wild *Acropora muricata*, from Heron Island on the Great Barrier Reef, (b) *Acropora* sp. from aquarium at Zoological Society of London (ZSL), (c) *Acropora* sp. showing brown jelly syndrome (BJS), (d) *Philaster* sp. found in corals exhibiting white syndrome at Heron Island (e) *Philaster* sp. found in corals exhibiting white syndrome in aquarium; (f) *Philaster* sp. found in corals exhibiting brown jelly syndrome in aquarium [note: these protozoa were previously miss-identified as *Helicostoma notatum*]. Sequence data provided analysis to genus level with phylogenetic analysis showing differences and similarities between these sequences (Figure 6). Morphologically these ciliates can be identified by a slender body, 60–200 x 20–60 µm in vivo, variable in outline from cylindrical to fusiform; anteriorly narrowed and conspicuously pointed. Length of the buccal field is ~ 40–50% of the body, cytostome is conspicuous and deeply sunk. Macronucleus band-like, twisted and positioned centrally along cell median with several micronuclei attached to it. One small, terminally located contractile vacuole. Approximately 50 somatic kineties composed of monokinetids, with cilia c. 7–10 m long; oral cilia c. 10–15 mm long; caudal cilium 12–15 mm in length. Paroral membrane L-shaped, on right of oral cavity, slightly oblique to main body axis. Locomotion by fast, spiral swimming while rotating irregularly about its main body axis, motionless for short periods when feeding. White Scale bars (a-c) = 1cm. Black Scale bars (d-f) = 10µm.

Bacterial diversity, DNA extraction, amplification and DGGE analysis

Bacterial partial 16S rRNA gene fragments were amplified using standard prokaryotic primers (357F) (5´-CCTACGGGAGGCAGCAG-3´) and (518R) (5'-ATTACCGCGGCTGCTGG-3'). The GC-rich sequence 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA GCA CGG GGG G-3' was incorporated in the forward primer 357 at its 5' end to prevent complete disassociation of the DNA fragments during DGGE. Thirty PCR cycles were performed at 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 1 min and a final extension at 72°C for 10 min (Sanchez *et al.* 2007). Three independent 10 µl PCR reactions were used, each containing 1.5 mM $MgCl_2$, 0.2 mM dNTP (PROMEGA), bovine serum albumin (BSA, 400 ng μl^{-1}), 0.5 mM of both the forward and reverse primers, 2.5 U of Taq DNA polymerase (QBiogene), incubation buffer, and 20 ng of template DNA (Siboni *et al.* 2007). These replicate PCR's for each sample were then combined and cleaned using QIAGEN QIAquick PCR purification kits, reducing the final volume to 15 µl in Sigma molecular grade H_2O. All reactions were performed using a Hybraid PCR Express thermal cycler. PCR products were verified by agarose gel electrophoresis (1.6% (w/v) agarose) with ethidium bromide staining and visualized using a UV transilluminator.

Sample Number	Species	Location	Health status	Rate of tissue loss (mm^2/day)
1	*Acropora pulchra*	HM	Healthy	NA
2	*Acropora formosa*	HM	Healthy	NA
3	*Acropora sp*	HM	Healthy	NA
4	*Acropora sp*	ZSL	Healthy	NA
5	*Pocilliopora damicornis*	NU	Healthy	NA
6	*Pocilliopora damicornis*	NU	Healthy	NA
7	*Acropora formosa*	NU	Healthy	NA
8	*Acropora formosa*	NU	Healthy	NA
9	*Pocilliopora damicornis*	HM	Healthy	NA
10	*Pocilliopora damicornis*	HM	Healthy	NA
11	*Acropora sp*	HM	WS[1]	0.2
12	*Acropora sp*	HM	WS[1]	0.2
13	*Seriatopora hystrix*	HM	WS[2]	0.2
14	*Montipora capricornis*	HM	WS[3]	0.5
15	*Pocilliopora damicornis*	HM	WS[1]	1.6
16	*Acropora sp*	ZSL	WS[1]	10
17	*Pocilliopora damicornis*	ZSL	WS[1]	10
18	*Euphyllia yaeyamnmesis*	ZSL	WS[1]	10
19	*Pocilliopora damicornis*	NU	WS[1]	10
20	*Pocilliopora damicornis*	NU	WS[1]	1.9
21	*Acropora sp*	ZSL	WS[1]	10
22	*Acropora sp*	ZSL	WS[1]	10
23	*Acropora sp*	ZSL	WS[1]	10
24	*Acropora sp*	ZSL	WS[1]	10
25	*Acropora sp*	ZSL	WS[1]	11
26	*Acropora sp*	NU	WS[1]	16
27	*Acropora formosa*	NU	WS[1]	10
28	*Hydnophora rigida*	HM	WS[1]	28
29	*Acropora sp*	ZSL	BJS	20
30	*Acropora sp*	Deep	BJS	15
31	*Acropora sp*	Deep	BJS	20

[1]*WS starting at the extremities;* [2]*WS starting along the branch;* [3]*WS patchy distribution*

Table 1. Sample set illustrating species and location utilised in this study, health status relates to described status of coral at time of samples. Healthy represent apparently healthy specimens, WS represent white syndrome disease signs, BJS brown jelly syndrome HM = Horniman Museum; ZSL = Zoological society of London; NU = Newcastle University, Deep = The Deep in Hull. Rates of tissue loss are estimates by personal observations in tank systems prior to transportation up to Newcastle for analysis and may not be accurate. Pers. obs. at ZSL showed an average advance rate of the tissue lesion of 10 mm^2 day^{-1} from the base, until ~10 cm from the tip then the rate would be accelerated and the coral would appear to 'shut down' and die off overnight ~100mm^2

DGGE was performed using the D-Code universal mutation detection system (Bio-Rad). Bacterial PCR products were resolved on 10 % (w/v) polyacrylamide gels that contained a 30–60 % denaturant gradient for 13 h at 60 °C and a constant voltage of 50 V. Gels were stained with a concentrated solution of 9 µl Sybr® Gold (Sigma) in 50 µl of 1X TAE poured directly onto the gel surface, covered and left in the dark for 20 min then further washed in 500 ml 1X TAE for 30 min and visualized using a UV transilluminator. Bacterial operational taxonomic units (OTUs), were defined from DGGE band-matching analysis using BioNumerics 3.5 (Applied Maths BVBA) following methods described by Guppy and Bythell (2006). Standard internal marker lanes were used to allow for gel-to-gel comparisons. Tolerance and optimisation for band-matching was set at 1%. OTUs of interest (those which explained the greatest differences/similarities between samples), were identified by sequence analysis. Bands were excised from DGGE gels, left overnight in Sigma molecular grade water, vacuum centrifuged, re-amplified with the appropriate primer set, labelled using Big Dye (Applied Biosystems) transformation sequence kits and sent to Genevision (Newcastle University, UK) for sequencing.

Ciliate diversity, DNA extraction, amplification and DGGE analysis

Single cell isolates

Corals were viewed under a binocular microscope and single cell isolates of ciliate morphotypes were sampled using a 10 µl pipette and stored in 1.5 ml micro-centrifuge tubes containing 100 µl EtOH at -20°C. DNA was extracted from the ethanol-fixed single isolates following a modified Chelex Extraction (Walsh *et al.* 1991). The sam-

ples were centrifuged for 2 min at 20,000 g and washed 3 times in sigma water with a centrifuge step between each wash. After the last wash 50 µl of a 5 % Chelex 100 (sigma) solution and 15 µl of proteinase K (20 mg/ml) were added to the cell isolate. Samples were incubated overnight in a water bath at 56°C, vortexed for 10 sec, boiled in a 100°C water bath for 10 min, vortexed again for 10-20 sec, and finally centrifuged for 3 min at 16,000 g; 40 µl of the supernatant was taken off and put in a fresh micro-centrifuge tube for use in subsequent PCR reactions.

PCR amplifications of single cell isolates were carried out using 20 µl reaction mixtures (final PCR buffer contained: 1 mM $MgCl_2$, and 1 U Taq DNA polymerase (QBiogene); 100 µM deoxynucleoside triphosphates; 0.2 µM of each of the forward and reverse primers; and 0.4 % bovine serum albumin, with 20 ng of template DNA. All reactions were performed using a Hybaid PCR Express thermal cycler. PCR products were analysed by agarose gel electrophoresis [1.6 % (w/v) agarose] with ethidium bromide staining and visualized using a UV transilluminator. A nested PCR was used to yield best results, initially with the universal 18S eukaryotic primer pairs f4617 (5'-TCCTGCCAGTAGTCATATGC-3') (T. Tengs *pers. commun.*) and r4618 (5'-TGATCCTTCTGCAGGTTCACCTAC-3'). PCR protocol was; 95°C for 3 min, then 40 cycles of (95°C for 30 sec, 55°C for 30s and 72°C for 90s); followed by 5 min at 72°C (Oldach *et al.* 2000). Then with internal ciliate specific primers 384f-cil (5'-YTBGATGGTAGTGTATTGGA-3') and 1147r-cil (5'-GACGGTATCTRATCG TCTTT-3'). PCR protocol was; 94°C for 5 min, then 30 cycles of (94°C for 45 sec, 55°C for 60 sec and 72°C for 90 sec); followed by 72°C for 7 min (Dopheide *et al.* 2008). Sequencing was carried out as above using Big Dye sequence kits and cleaned with an EtOH precipitation method and sequenced at Genius limited, Newcastle University.

Ciliate DGGE

The same coral samples (as used for the bacterial analysis) were analysed for their ciliate diversity. Partial ciliate 18S rRNA gene ribotypes were amplified with a single-round PCR approach (Jousset *et al.* 2010). Three replicate PCR reactions were carried out as above (final PCR buffer contained: 1 mM $MgCl_2$, and 1 U Taq DNA polymerase (QBiogene); 100 µM dNTPs; 0.2 µM of each of the forward and reverse primers; and 0.4% BSA, with 20 ng of template DNA. Primers Cil-f (5'-TGGTAGTGTATTGGACWACCA-3') with a 36-bp GC clamp (Muyzer and Smalla 1998) attached to the 5' end and CilDGGE-r (5'-TGAAAACATCCTTGGCAAATG-3') were used. Initial denaturation was at 94°C for 5 min, followed by 26 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min and a final elongation of 10 min at 72°C to reduce double bands in the DGGE patterns (Janse *et al.* 2004). PCR products of the 3 replicate samples were combined and subjected to DGGE on a D-code system (Bio-Rad) with 0.75 mm thick 6 % polyacrylamide gels in 1 X TAE buffer. Electrophoresis was carried out for 16 h at 60°C and 50 V in a linear 32 to 42 % deionised formamide denaturing gradient. Gels were stained as above and OTUs of interest were similarly identified by band excision and sequencing.

Quantitative PCR (qPCR) targeting Vibrio sp.

Quantitative PCR (qPCR) was conducted on an Engine Opticon® 2 system in order to test whether *Vibrio* sp. relative 16S rRNA abundance differed between a random subset of the diseased samples (n = 10) and healthy coral samples (n = 10). qPCR assays were standardised using a serial dilution (10^9 – 10^1 CFU ml^{-1}; 3 replicates per dilution) of pure cultures of *Vibrio harveyi* (NR043165) and run on the qPCR machine to determine threshold cycles. *Vibrio*-specific primers 567F (5'-GGCGTAAAGCGCATGCAGGT-3') and 680R (5'-GAAATTCTACCCCCCTCTACAG-3') (Thompson *et al.* 2004; Sweet *et al.* 2010), were used for all samples. qPCR reaction mixtures totalled 25 µl and consisted of 12.5 µl of 2X Quantitect® Sybr® Green 1 supermix (Qiagen), 1.25 µl each of 0.5 mM forward and reverse primers, 50 ng DNA and 9.5 µl Sigma molecular grade water. Each set of samples included a negative control, in which water was substituted for the DNA sample. qPCR was performed with an initial activation step of 15 min at 95°C, followed by 39 cycles (94°C for 15 sec, 58°C for 30 sec, primer annealing at 58°C for 30 sec). The fluorescent product was detected after each extension. Following amplification, melting temperature analysis of PCR products was performed to determine the specificity of the PCR. The melting curves were obtained by slow heating at 0.5°C sec^{-1} increments from 50 to 90°C, with continuous fluorescence recording.

Total bacterial abundance

To estimate bacterial abundance, 1000 µl of tissue slurry was filtered through a 0.22 µm black polycarbonate filter and fixed with 100 µl of paraformaldehyde until analysis (Fuhrman *et al.* 2008). These filters were stained with 100 µl DAPI solution (final concentration 5 µg ml^{-1}) for 10 min, rinsed with Phosphate Buffer Solution (Yu *et al.* 1995; Weinbauer *et al.* 1998; Yamaguchi *et al.* 2007), and viewed under epifluorescence microscopy using a DAPI-specific filter set. Counts on 50 fields of view (FOV), were taken using an automatic cell counter (Cell C; (Selinummi *et al.* 2005). The parameters were set to exclude any objects smaller than 0.03 μm^2 and anything larger than 0.7 μm^2. Counts were scaled up to the total area of the filter and calculated to give total bacterial abundance per volume of tissue on the diseased corals (cells cm^3). Total amount of diseased tissue rather than complete coral nubbin surface area was used to account for the varying amount of tissue on the diseased samples as this could not be standardised at time of collection. Counts of n = 3 tissue subsamples were taken from each coral and averaged to provide a cell density per sample.

Statistical analysis

Analysis of Similarity (ANOSIM) tests based on Bray-Curtis similarities (Clarke and Warwick 2001) were used to test for differences in the bacterial 16S and ciliate 18S rRNA gene DGGE profiles associated with diseased and healthy corals. A non-metric multidimensional scaling (MDS) analysis was used to represent each sample type on a 2-D plot (Clarke and Warwick 2001). An analysis of contribution to similarities (SIMPER) based on DGGE analysis with both relative band intensity and presence absence was performed to determine which 16S and 18S rRNA gene OTUs best explained dissimilarities among sample types that were statistically different. The abundance of bacteria (total bacterial counts) was compared between healthy and diseased samples with a one-way ANOVA. Data met the assumptions of normality and equality of variances (Anderson Darling and Levene's tests respectively). For qPCR a standard curve delineating threshold (CT) values of *V. harveyi* against number of *V. harveyi* (CFU ml^{-1}) in pure cultures gave a standard curve (R^2 = 0.996) allowing quantification of the assay. Calculations were then based on 1 CFU relating to a genome copy. One way ANOVA was used to compare between healthy and diseased samples for *Vibrio* fold difference.

Results

Significant differences based on profiling of bacterial communities using DGGE analysis (PERMANOVA, R = 0.494, p = 0.001), were found between the bacterial 16S rRNA gene diversity of healthy and diseased coral samples (Fig 2, 3). Within healthy coral samples, there were no significant differences between the three aquariums where samples were sourced from, or between species of coral (two-way crossed ANOSIM, R = 0.29, p = 0.2 and R = 0.187, p = 0.28, respectively). Similarly no significant differences were detected between diseased samples with different rates of progression and disease type (WS or BJS) (two-way crossed ANOSIM, R = 0.19, p = 0.09 and R = 0.22, p = 0.12 respectively, Fig 3a,b).

There was a significant difference in bacterial abundance associated with healthy versus diseased corals (ANOVA, df = 1, F = 5.15, p = 0.03), with mean bacterial abundance increasing in diseased corals (6.1 ± 0.52 (SD) x 10^7 cells cm^{-3}), compared to that of healthy samples (3.5 ± 0.23 (SD) x 10^7 cells cm^{-3}) (Fig 4a). *Vibrio* abundance also

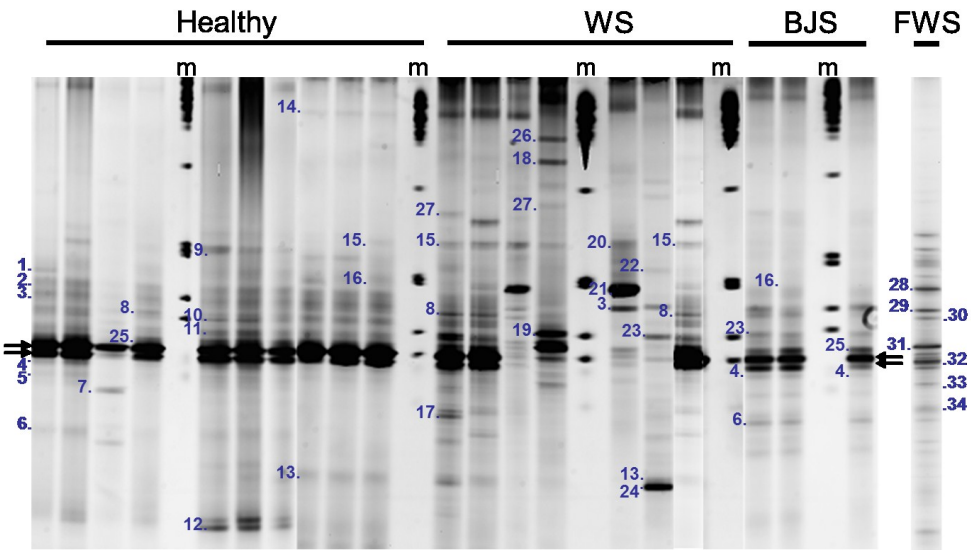

Figure 2. DGGE analysis of bacterial 16S rRNA gene diversity from healthy and diseased corals. Healthy = corals showing no signs of distress or symptoms of disease at time of collection; WS = white syndrome collected from aquariums, corals showing signs of some form of tissue necrosis similar to that known as rapid tissue necrosis or white syndrome. FWS = field white syndrome from Heron Island as reported in Sweet and Bythell 2012, used as a reference to show similarities and differences between those in aquarium and those in the wild. Arrows indicate corals symbiotic algae, *Symbiodinium* sp. Band numbers (1 – 27 in blue) relates to ribotypes excised from the cell and sequenced (Table 2). Intermittent marker lanes (m) allowed gel to gel comparison using the software BioNumerics.

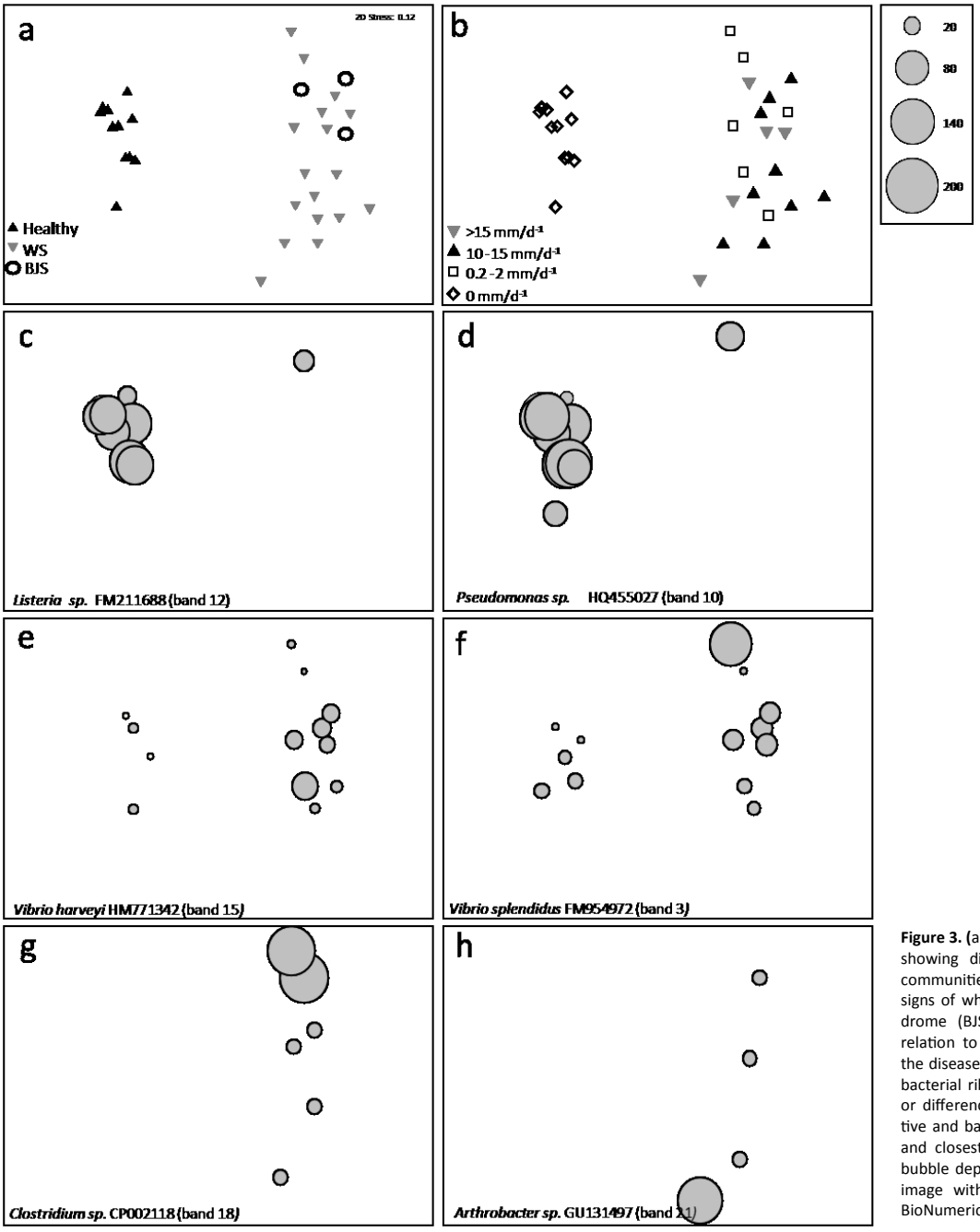

Figure 3. (a) Multidimensional scaling (MDS) plot showing differences in 16S rRNA gene bacterial communities from healthy corals to corals showing signs of white syndrome (WS) and brown jelly syndrome (BJS). (b) shows grouping of samples in relation to variation in their lesion progression of the disease (mm per day) (c-h) show 16S rRNA gene bacterial ribotypes that caused greatest similarities or differences between sample types (closest relative and band no in relation to DGGE image [Fig 2] and closest match on GenBank [Table 2]. Size of bubble depicts intensity of band/ribotype on DGGE image within individual samples calculated using BioNumerics.

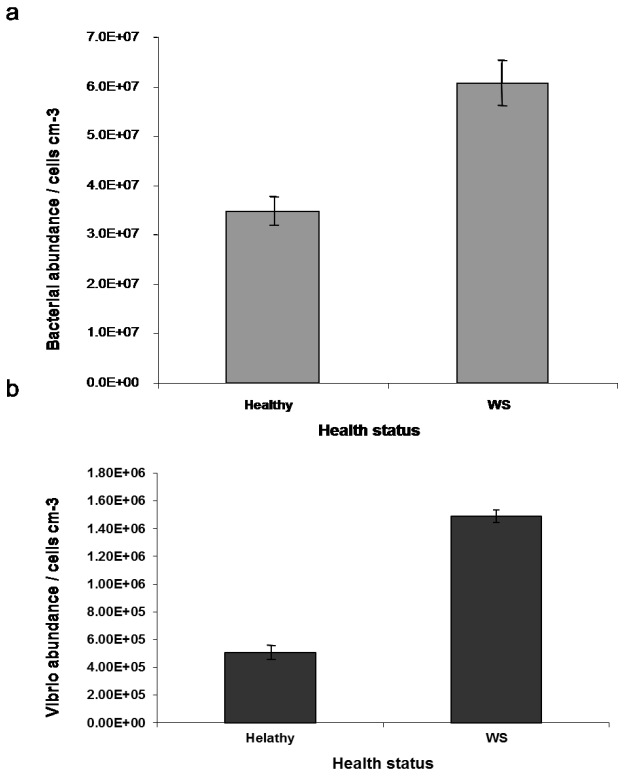

Figure 4. (a) Total bacterial abundance of healthy and diseased corals within aquarium, acquired from filtered tissue slurry and DAPI staining, counts made using the automatic cell counter Cell C; (b) total relative *Vibrio* sp. per cm^3 of tissue present within healthy and diseased coral samples, acquired from quantitative PCR (see materials and methods) for ten replicate qPCR runs for both healthy and diseased samples. Error bars represent SE for collective mean.

increased significantly (ANOVA, df = 1, F = 4.46, p = 0.043) in diseased coral compared to healthy (1.5 ± 0.48 (SD) x 10^6 cell cm^{-3} and 5.3 ± 0.37 (SD) x 10^5 cells cm^{-3} respectively) (Fig 4b).

There were several bacterial ribotypes (a *Pseudomonas* sp. GenBank closest relative HQ455027 and a *Listeria* sp. GenBank closest relative FM211688) which reduced in dominance in diseased tissues compared to healthy samples (Fig 2 and 3c,d). Whilst two other ribotypes were completely absent from diseased samples, including a *Pelobacter* sp. (GenBank closest relative CP000482) and a *Acidiobacterium* sp. (GenBank closest relative EF076073) (Table 2). Several bacterial ribotypes increased in relative 16S rRNA gene abundance in some diseased samples including ribotypes similar to a *Pseudovibrio* sp. (GenBank closest relative GU826592), a *Cyanobacterium* sp. (GenBank closest relative HQ230344), a *Arcobacter* sp. (GenBank closest relative HM584709) and two *Vibrio* sp. (GenBank closest relative HM771342 and FM954972; Fig 3e and f respectively). Three bacterial ribotypes were absent from healthy specimens and dominant in some diseased samples, including a *Clostridium* sp. (GenBank closest relative CP002118; Fig 3g), a *Arthrobacter* sp. (GenBank closest relative GU131497; Fig 3h) and a *Microbacterium* sp. (GenBank closest relative EU249984) (Fig 2 and 3, Table 2). However, there were no bacterial ribotypes consistently present in all cases of disease (Fig 2).

Ciliates were only observed on diseased tissues and completely absent in healthy corals. This observation was supported by 18S rRNA gene analysis which showed a diversity of ciliate ribotypes in diseased samples and produced no PCR product in healthy samples (Fig 5). At least 7 ciliate ribotypes were consistently present in all diseased corals. These included ribotypes similar to a *Pseudokeronopsis* sp. (GenBank closest relative HQ228545), a *Aspidisca* sp. (GenBank closest relative AF305625), a *Philaster* sp. (GenBank closest relative FJ648350), a *Glauconema* sp. (GQ214552), a *Paradiscocephalus* sp. (GenBank closest relative EU684746), a *Licnophora* sp.

			Abundance (band intensity)		Average dissimilarity 69.95%	
DGGE band ID	Species ID	Closest relative (% match)	Healthy	Diseased	Contribution %	Accumulative contribution (%)
10	*Pseudomonas sp.*	HQ455027 (100%)	4.13	0.38	8.28	8.28
12	*Listeria sp.*	FM211688 (100%)	3.48	0.28	7.15	15.43
26	*Pseudovibrio sp.*	GU826592 (99%)	0.35	3.25	6.25	21.68
23	*Cyanobacterium*	HM535495 (99%)	0.52	2.21	4.07	25.75
NA	Unknown	NA	1.27	1.43	3.77	29.51
2	*Pelobacter sp.*	CP000482 (99%)	1.77	0	3.74	33.25
4	*Oscillatoriales cyanobacterium*	GU967420 (95%)	2.47	2.41	3.58	36.83
16	*cyanobacterium*	EF577468 (96%)	1.3	1.29	3.32	40.15
25	*Arcobacter sp.*	HM584709 (96%)	0.78	1.41	3.31	43.47
24	*Clostridium sp.*	AY712079 (97%)	1.99	3.43	3.29	46.76
15	*Vibrio harveyi*	HM771342 (96%)	0.67	1.43	3.28	50.04
3	*Vibrio splendidus*	FM954972 (100%)	0.81	1.31	3.23	53.27
18	*Clostridium sp.*	CP002118 (96%)	0	1.49	3.07	56.34
11	*Campylobacter sp.*	HM462470 (97%)	2.2	1.54	2.39	58.73
NA	Unknown	NA	0.58	0.94	2.11	60.84
1	*Stenotrophomonas sp.*	HM153430 (97%)	0.9	0.78	2.07	62.91
9	*Acidobacterium sp.*	EF076073 (100%)	0.98	0	2.05	64.96
6	*Chloroflexi sp.*	DQ330160 (98%)	0.72	0.39	1.96	66.91
NA	Unknown	NA	0.91	0.69	1.84	68.75
NA	Unknown	NA	0.05	0.9	1.83	70.58
27	*Marinobacter sp.*	HM141532 (98%)	0.92	0.77	1.82	72.4
13	*Pectobacterium sp.*	DQ123811 (99%)	0.43	0.69	1.81	74.21
NA	Unknown	NA	0.38	0.77	1.67	75.88
22	*Verrucomicrobiae sp.*	FN646737 (93%)	0.53	0.52	1.67	77.55
21	*Arthrobacter sp.*	GU131497 (96%)	0	0.78	1.64	79.18
19	*Microbacterium sp.*	EU249984 (98%)	0	0.79	1.64	80.82
8	Uncultured bacterium sp.	GU118719 (100%)	0.67	0.26	1.6	82.42
NA	Unknown	NA	0.13	0.77	1.57	83.99
7	*Gamma proteobacterium*	EF215814 (100%)	0.37	0.42	1.52	85.51
17	*Arcobacter sp.*	FR675876 (100%)	0.19	0.63	1.51	87.02
NA	Unknown	NA	0.15	0.69	1.5	88.52
NA	Unknown	NA	0.45	0.43	1.47	89.99
5	*Arcobacter sp.*	HQ317346 (96%)	0.49	0.25	1.32	91.31

Table 2. Closest match (GenBank ID) and identification of bacterial species from healthy and diseased corals in aquarium, sequenced from denaturing gradient gel electrophoresis (DGGE) bands. Out of a total of 44 operational taxonomic units (OTUs), 33 are represented in this table which account for 91.3% of variance between sample types. Relative abundance measurements are based on BioNumerics presence/absence and band intensity data

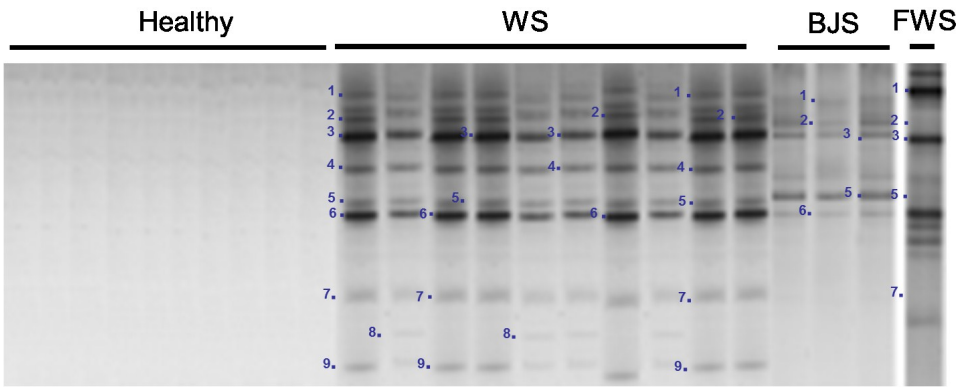

Figure 5. DGGE analysis of ciliate 18S rRNA gene diversity from healthy and diseased corals. Healthy = corals showing no signs of distress or symptoms of disease at time of collection; WS = white syndrome from aquarium, corals showing signs of some form of tissue necrosis similar to that known as rapid tissue necrosis or white syndrome. FWS = Field white syndrome from Heron Island as reported in Sweet and Bythell (2012), used as a reference to show similarities and differences between those in aquarium and those in the wild. Band numbers (1–9 in blue) relate to ribotypes excised from the cell and sequenced. 1 = *Pseudocarnopsis* sp. (HQ228545); 2 = *Aspidisca steini* (AF305625), 3 = *Philaster* sp. (FJ648350), 4 = *Hanseniaspara* sp. (JF306024), 5 = *Glauconema* sp. (GQ214552), 6 = *Parasiscocephalus elongates*, 7 = *Holostica diadementa* (DQ059583), 8 = *Licnophora lyngbycola* (DQ445606) and 9 = *Chromodorina* germonica (AY854207).

(GenBank closest relative DQ445606) and a *Holosticha* sp. (GenBank closest relative DQ059583). All morphotypes detected visually and identified by sequencing single cell isolates were also detected by DGGE analysis of the ciliate community. Five of the seven ribotypes identified in WS samples matched (with >99% similarity) to those recently identified in WS in the wild (Sweet and Bythell 2012, Fig 5). These included the *Pseudokeronopsis* sp., *Aspidisca* sp., *Philaster* sp., *Glauconema* sp. and *Holosticha* sp. One DGGE band in the aquarium samples (Band 4 in Fig 5) was identified as a fungus, *Hanseniaspara* sp. (GenBank closest relative JF306024), and another (Band 9 in Fig 5) was identified as a nematode similar to *Chromodorina* sp. (GenBank closest relative AY854207), indicative of a limited amount of non-specific PCR amplification with these primers.

Only one ciliate type was observed to ingest coral tissue, as evident from the presence of coral symbiotic algae within the cell (Fig 1 d-f). This was commonly the most abundant ciliate observed and was found consistently in all WS and BJS samples. It was identified from single cell isolates as 99% similar over 549 base pairs to *Philaster digitformis* (FJ648350) and was morphologically similar to *Porpostoma notatum* (=*notate*), (Song 2000), *P. guamense* (Lobban *et al.* 2011) and *Helicostoma notatum* (the ciliate associated with BJS reviewed in Sweet *et al.* 2011a). However, single cell sequences obtained from this morphotype in aquarium disease samples were distinct (92% sequence similarity over 549 bp) from the recently submitted sequence for *Porpostoma notatum* (GenBank closest relative HM236335). Sequence data is currently lacking for the only other *Porpostoma* species reported, *Porpostoma guamense* so comparison with this species on a genetic level could not be done. Two unique GenBank accession numbers have been submitted for the morphotype in this study, JF831358 for ciliates acquired from corals with WS and JF831359 for ciliates associated with BJS. These WS and BJS sequences were closely related (>99% over 549 bp) to ribotypes recently identified in wild corals with WS (GenBank closest relative HQ204545) and Brown Band Disease (GenBank closest relative HQ204546), varying by only 2 bp over 549 (Sweet and Bythell 2012; Fig 6). Thus, the evidence strongly suggests that this same ciliate is the dominant member of WS-associated communities in both aquarium and wild samples and is also a dominant member of BJS and BrB communities (this study and Sweet and Bythell 2012).

Discussion

There was a significant difference between bacterial 16S rRNA gene diversity of healthy and diseased coral samples and a general increase in bacterial load within diseased samples, a result consistent with previous findings (Luna *et al.* 2007; Sussman *et al.* 2008; Ainsworth *et al.* 2010; Luna *et al.* 2010). In previous studies specific bacteria have been proposed as single causal agents to certain coral diseases such as WS. The most commonly referred to are from the genus *Vibrio* (Luna *et al.* 2007; Sussman *et al.* 2008; Luna *et al.* 2010). However, in this study there were no single dominant bacterial ribotypes consistently present within all diseased samples. This supports the report by Willis *et al.* (2004), in which they defined WS as a group of unidentified diseases occurring in Indo-Pacific corals with unknown etiology. Two *Vibrio* sp. and a *Pseudovibrio* sp. were found to increase in abundance within diseased specimens, making them potential candidates for pathogenesis (Sweet and Bythell 2012). However, the two *Vibrio* sp. were also detected in healthy as well as diseased samples. Several other potential pathogens were also shown to increase in relative 16S rRNA gene abundance in individual diseased samples, including ribotypes similar to; a *Cyanobacteria* sp., a *Arcobacter* sp., a *Clostridium* sp., a *Arthrobacter* sp. and a *Microbacterium* sp., however none of these specific bacteria were consistently detected in all diseased samples.

If WS was simply a case of a specific single bacterial pathogen and providing that the samples were taken at the same stage of disease progression, when casual agents would be in high abundance and high activity, you would expect to see a single dominant ribotype consistent across all samples exhibiting signs of this disease. It is therefore likely that WS is caused by an initial systemic infection by any number of potential bacterial pathogens depending on which are present when the coral becomes stressed. In addition, it should be possible to co-localise these bacterial populations with histopathology. There should be an increase in bacterial populations at the disease lesion interface and/or classic signs of bacterial induced necrosis, which up to now has not been possible (Ainsworth *et al.* 2007; Work and Aeby 2011).

In contrast, the consistent presence of the same ciliate, identified as similar to *Philaster digitformis* (FJ648350), in all diseased samples exhibiting signs of WS and those from samples exhibiting BJS, yet absent in the healthy specimens, suggests that this ciliate is an important and regularly detectable associate of the disease. Observations of the ingestion of coral tissues, and presence of coral endosymbiotic algae within the ciliate suggests that either these ciliates are directly involved in pathogenesis or alternatively they are simply associated with the necrotic tissue of the disease. Regardless, these ciliates are clearly important in the pathology of these aquarium diseases (namely the sharp band of denuded skeleton adjacent to apparently healthy tissue) a result supporting that recently found in the wild (Sweet and Bythell 2012). The ciliate identified as being involved in pathogenesis in these aquarium diseases is >99% similar to ciliates also consistently identified in both WS (HQ204545) and BrB (HQ204546) in the wild (Sweet and Bythell 2012). Several other ciliates previously identified in WS and BrB in the wild were also detected with the aquaria however were not shown to ingest coral tissues and are likely secondary colonisers. Interestingly, the dominant ciliate of BrB, first identified by Bourne *et al.* (2008), and also involved in coral tissue feeding in the wild diseases (Sweet and Bythell 2012), was absent from the aquarium diseases, suggesting that it not a necessary component of the WS pathology.

Although disease causation cannot be inferred using a purely culture independent approach, the observations of histophagy (Ainsworth *et al.* 2007; Work and Aeby 2011), together with similari-

ties of ciliate communities in aquarium and wild corals displaying similar disease signs has led us to confirm our hypotheses reported in Sweet and Bythell (2012). In short, either; (i) opportunistic pathogenic bacterial species such as the widely-reported vibrios are the primary agents, invading healthy tissues and leading to an impaired physiological condition which allows ciliate communities to invade and proliferate at the disease lesion. Or, alternatively (ii) ciliates are the causal agents and the bacterial agents identified are either non-specific pathogens infecting the tissues that have been compromised by ciliate histophagy or are invading the dead and decomposing tissues at the lesion interface or the skeletal surfaces immediately adjacent to it. A further hypothesis can also be inferred in that the corals physiological condition is severely impaired due to either environmental stress or for example infection from other organisms not investigated in this study e.g. viruses (Davy *et al.* 2006; Marhaver *et al.* 2008) and/or fungi (Lecampionalsumard *et al.* 1995). This in turn would then allow for secondary invasion scenarios from both bacteria and ciliates.

Since potential bacterial pathogens previously linked to coral disease have routinely been detected in healthy corals in this and many other studies (Bourne and Munn 2005; Klaus *et al.* 2005; Gil-Agudelo *et al.* 2007; Arboleda and Reichardt 2009; Kvennefors *et al.* 2010; Luna *et al.* 2010), it is essential that studies assessing disease causation by techniques such as pure culture inoculations, control for the inadvertent increase in relative abundance of other potential pathogens. In our own experimental aquarium facilities, the ciliate type implicated in pathogenesis in this study (JF831359), was prevalent in aquarium samples within one week of initial set up (pers. obs.). Since we show here that these ciliates are absent from non-diseased coral, we strongly recommend that future studies addressing Koch's postulates, simultaneously test for the presence of ciliates via light microscopy and molecular screening to ensure that the stress of applying the inoculate does not inadvertently promote these or other potential pathogens, which are apparently ubiquitous in the field and in experimental aquaria.

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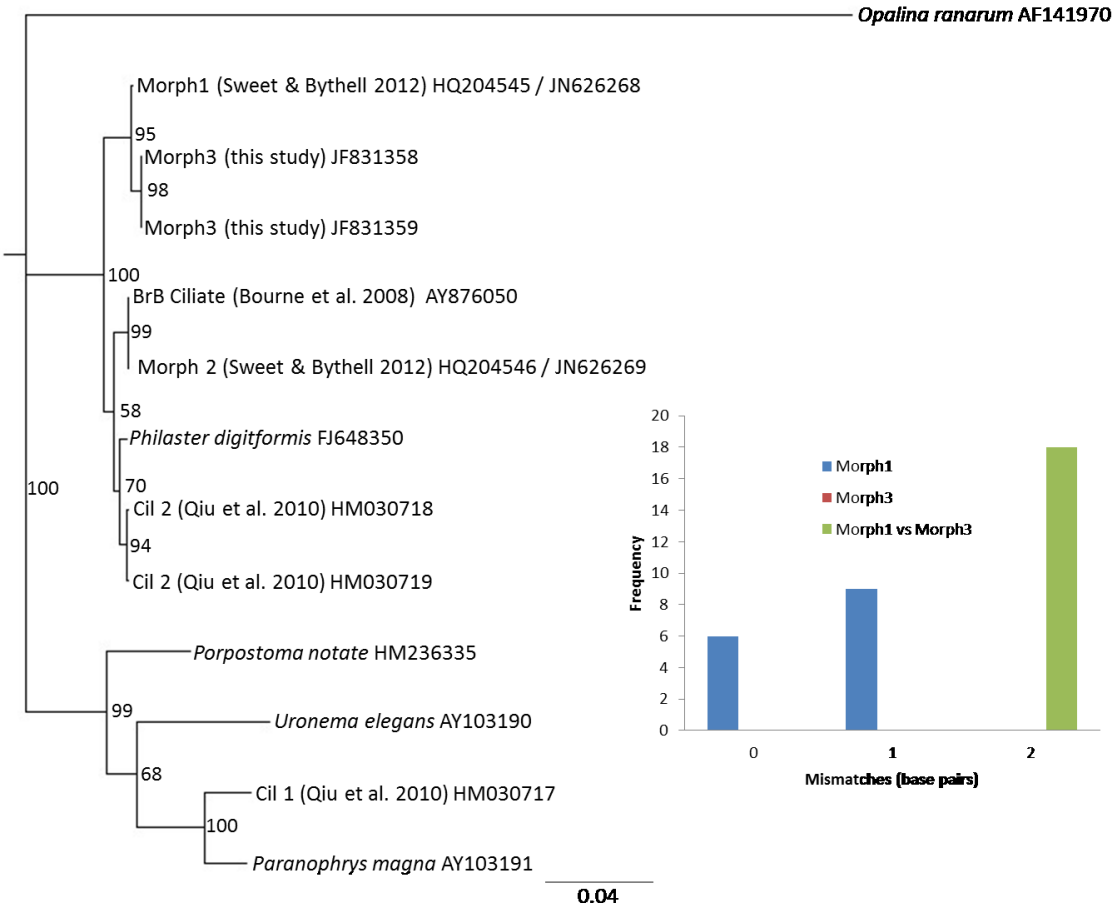

Figure 6. Neighbour-joining consensus tree of partial 18S rRNA gene sequences of 16 samples of ciliates found within corals exhibiting both brown jelly syndrome and white syndrome within UK aquariums and wild type ciliates found in corals exhibiting signs of white syndrome from Heron Island. Sequences were aligned in Clustal W2, using an IUB cost matrix with a gap open cost of 15 and a gap extend cost of 7. A neighbour-joining consensus tree (1000x re-sampling) was constructed in genius Pro 5.0 using Tamura and Nei (1993) genetic distance model with an opalinid protest, *Opalina ranarium* (AF149070), as the outgroup. Insert histogram shows sequence mismatch frequencies within and between sequences of Morph 1 and 3.

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