Ciliate Communities Consistently Associated with Coral Diseases

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Ciliate communities consistently associated with coral diseases

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ABSTRACT

Incidences of coral disease are increasing. Most studies which focus on diseases in these organisms routinely assess variations in bacterial associates. However, other microorganism groups such as viruses, fungi and protozoa are only recently starting to receive attention. This study aimed at assessing the diversity of ciliates associated with coral diseases over a wide geographical range. Here we show that a wide variety of ciliates are associated with all nine coral diseases assessed. Many of these ciliates such as *Trochilia petrani* and *Glauconema trihymene* feed on the bacteria which are likely colonizing the bare skeleton exposed by the advancing disease lesion or the necrotic tissue itself. Others such as *Pseudokeronopsis* and *Licnophora macfarlandi* are common predators of other protozoans and will be attracted by the increase in other ciliate species to the lesion interface. However, a few ciliate species (namely *Varistrombidium kielum*, *Philaster lucinda*, *Philaster guamense*, a *Euplotes* sp., a *Trachelotractus* sp. and a *Condylostoma* sp.) appear to harbor symbiotic algae, potentially from the coral themselves, a result which may indicate that they play some role in the disease pathology at the very least. Although, from this study alone we are not able to discern what roles any of these ciliates play in disease causation, the consistent presence of such communities with disease lesion interfaces warrants further investigation.

1. Introduction

Historically, most coral diseases (specifically those showing aspects of tissue loss) have been associated with numerous pathogenic bacteria (Ben-Haim and Rosenberg, 2002; Cervino et al., 2008; Frias-Lopez et al., 2003; Kushmaro et al., 2001; Luna et al., 2010; Patterson et al., 2002; Richardson et al., 1998; Sussman et al., 2008). However, attention is now turning to other microorganisms such as fungi, viruses and ciliates (Katz et al., 2014; Sweet and Bythell, 2012; Sweet et al., 2014). The first coral disease associated with ciliates for example was Skeleton-Eroding Band (SEB), which was initially described in 2001 (Antonius and Lipscomb, 2001). Characterized by a speckled black band associated with the lesion interface, the disease is thought to be caused by the folliculinid ciliate, *Halofolliculina corallasia* (Winkler et al., 2004). Three years later another coral disease Brown Band Disease (BrB) was described and associated with the ciliate *Philaster guamense*, at the time described as *Porpostoma guamense* (Lobban et al., 2011; Willis et al., 2004). Over in the Caribbean, two years after BrB was first described ciliates similar to those associated with SEB (*Halofolliculina*) were reported affecting over 26 Caribbean reef-building coral species, and the term Caribbean Ciliate Infection (CCI) was coined (Croquer et al., 2006a). Six years later still and White Syndrome (WS), the most prevalent disease sign around the world has also been shown to have a diverse ciliate community associated with the lesion interface (Sweet and Bythell, 2012). More recently, White Band Disease (WBD), White Plague, Brown Jelly Syndrome and another BrB-like syndrome in the Caribbean have all been described as having ciliates associated with the disease signs (Randall et al., 2014; Sweet et al., 2014). Although there are an increasing number of studies linking different ciliate species to specific coral disease states, there are currently no published studies which highlight exactly how such ciliates cause disease. Furthermore, there have been no controlled inoculation experiments identifying if indeed these proposed pathogens are the primary causal agents. This has led to the general belief that many if not all these coral associated ciliates are opportunistic, eating the dead and dying tissue caused by another as yet unknown pathogenic agent. In fact, two recent studies have highlighted that this is actually likely the case, at least for WBD in the Caribbean and WS in the Indo-Pacific (Sweet and Bythell, 2015; Sweet et al., 2014). However, regardless of the specific role of ciliates (which remains to be determined), a first step in understanding their importance in coral disease would be to assess the community associated with different disease states globally. Here, we therefore aim to provide an initial baseline assessment of the ciliate communities associated with nine dominant coral diseases located in both the Indo-Pacific and the Caribbean. Although every care was taken to ensure that a representative sample was taken, we by no means guarantee that every ciliate species has been described here in this study and it remains highly likely

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that more species will be associated with these diseases as further studies assess for them.

2. Methods

2.1. Coral samples

Any coral disease which was present during the time of sampling was collected. Coral from seven Genera were sampled (*Acropora cervicornis*, *Acropora muricata*, *Acropora aspera*, *Pocillopora damicornis*, *Colpophyllia natans*, *Orbicella annularis*, *Porites lutea*, *Goniopora djiboutiensis* and *Diploria strigosa*), from nine geographical locations (Venezuela, Colombia, Australia, the Solomon Islands, Fiji, La Reunion, Moyonette, South Africa and the Maldives) (Table 1). The diseases included; White Syndrome (WS), White Plague Disease (WPD), White Band Disease (WBD), *Porites* White Patch Syndrome (PWPS), Caribbean Ciliate Infection (CCI), Skeletal Eroding Band (SEB), Caribbean Yellow Band Disease (YBD), Brown Band Disease (BrB) and Black Band Disease (BBD) (Fig. 1). Signs associated with each disease were characterized as outlined by the Global Coral Disease Database (coraldisease.org/diseases). Any coral with signs of disease were tagged and monitored in situ to assess disease progression before sampling. Coral fragments were collected only from tagged corals with signs of progression. Fragments (which included the disease lesion interface and 1 cm of healthy tissue) were transported to the field laboratories and the ciliate community assessed under light microscopy. Single cell isolates (minimum of three per ciliate species) were taken for both morphological and genetic identification as described below. In addition, coral fragments containing only apparently healthy tissue were collected from the same colony as that of the disease tissue fragment. Apparently healthy fragments were similarly assessed for the presence of ciliates.

2.2. Identification of ciliate diversity

2.2.1. Microscopic observation and characterization of the dominant ciliates

Microscopic and behavioral observations of associated ciliate species were made using an Olympus SZX7 binocular microscope and Olympus LG-PS2 fiber-optic light source immediately after collecting the coral samples from the field. During assessment of the ciliate community, any corals not being immediately assessed were stored in separate aquaria with free flowing filtered sea water until they were placed under the microscopes. All corals were analyzed within 2 h from initial sampling. Still images were captured using a QImaging Micropublisher 3.3 camera and Q-Capture v6 imaging software. Higher magnification images were obtained using an Olympus BX51 compound microscope. The images were compared to morphological descriptions in previous studies (Carey, 1992; Croquer et al., 2006b; Lee et al., 2000; Page et al., 2008; Shimano et al., 2008; Song, 2000; Sweet and Bythell, 2012), alongside the use of a dichotomous key in the 'Illustrated Guide to the Protozoa' (Lee et al., 2000). Morphological characteristics provided a further means of distinguishing ciliate morphotypes to confirm that our sequence data (see below) matched previously identified protozoan species. Features such as kinetosomal make-up and oral infraciliary structures such as the AZM (Adoral Zone of Membranelles) are highly conserved and together with organelle distribution, size, shape and color can be routinely used for distinguishing genera (Lee et al., 2000). A minimum of 30 min was spent assessing the ciliate community associated with each sample. Where possible, replicate samples of the disease were assessed from the same location and the same coral species. Unfortunately, standardization of samples was impossible as sampling was opportunistic and depended on the coral disease encountered. This resulted in an uneven representation of the different coral diseases assessed and the number and species of corals sampled (Table 1). Due to

Table 1
Illustrates the common disease name, the location sampled, the number of replicates, which coral species were sampled and the ciliates present on the coral diseases reported in this study.

Disease name	Location sampled	Number of corals sampled and from which species	Ciliates species present
White Syndrome (WS)	Australia, Solomons, Fiji, Maldives, UK Aquaria	N = 40 *Acropora muricata* (15) *Acropora aspera* (15) *Pocillopora damicornis* (10)	*Diophrys* sp., *Holosticha diademata*, *Varistrombidium kielum*, *Protocruzia adherens*, *Trochilioides recta*, *Uronema heteromarinum*, *Philaster lucinda*, *Philaster guamense*, *Trochilia petrani*, *Glauconema trihymene*, *Litonotus pictus*, *Euplotes* sp., *Aspidisca* sp., *Pseudokeronopsis* sp., *Hartmannula derouxi*, *Licnophora macfarlandi*, *Dysteria derouxi*, *Hemigastrostyla enigmatica*, *Chaenea vorax*, *Acineta* sp.
Brown Band Disease (BRB)	Australia	N = 4 *Acropora muricata*	*Diophrys* sp., *Varistrombidium kielum*, *Philaster lucinda*, *Philaster guamense*, *Glauconema trihymene*, *Euplotes* sp., *Pseudokeronopsis* sp., *Holosticha diademata*
Porites White Patch Syndrome (PWPS)	Moyonette, Reunion, South Africa	N = 9 *Porites lutea*	*Holosticha diademata*, *Varistrombidium kielum*, *Uronema heteromarinum*, *Philaster lucinda*, *Dysteria derouxi*, *Paracineta limbata*
White Plaque (WP)	Venezuela, Columbia	N = 8 *Colpophyllia natans* (4) *Orbicella annularis* (4)	*Holosticha diademata*, *Varistrombidium kielum*, *Protocruzia adherens*, *Trochilioides recta*, *Uronema heteromarinum*, *Philaster lucinda*, *Licnophora macfarlandi*, *Anteholosticha* sp., *Cryptocaryon* sp., *Dysteria derouxi*, *Paracineta limbata*, *Chaenea vorax*, *Acineta* sp., *Suctoria* sp.
White Band (WB)	Venezuela, Columbia	N = 8 *Acropora cervicornis*	*Varistrombidium kielum*, *Protocruzia adherens*, *Trochilioides recta*, *Philaster lucinda*, *Trochilia petrani*, *Glauconema trihymene*, *Pseudokeronopsis* sp., *Licnophora macfarlandi*, *Anteholosticha* sp., *Dysteria derouxi*, *Paracineta limbata*, *Trachelotractus* sp., *Chaenea vorax*
Skeletal Eroding Band (SEB)	Moyonette, Reunion, South Africa	N = 4 *Acropora muricata*	*Holosticha diademata*, *Varistrombidium kielum*, *Trochilioides recta*, *Philaster lucinda*, *Licnophora macfarlandi*, *Halofolliculina corallasia*, *Dysteria derouxi*, *Paracineta limbata*, *Chaenea vorax*, *Condylostoma* sp.
Caribbean Ciliate Infections (CCI)	Venezuela	N = 3 *Acropora cervicornis*	*Philaster lucinda*, *Halofolliculina corallasia*, *Suctoria* sp.
Black Band Disease	Venezuela, Moyonette, Maldives, Reunion, South Africa	N = 18 *Diploria strigosa* (4) *Diploria* sp. (2) *Orbicella annularis* (3) *Colpophyllia natans* (2) *Acropora muricata* (3) *Porites lutea* (2) *Goniopora djiboutiensis* (2)	*Holosticha diademata*, *Protocruzia adherens*, *Philaster lucinda*, *Chaenea vorax*, *Suctoria* sp.
Caribbean Yellow Band Disease	Venezuela	N = 8 *Montastraea annularis*	*Holosticha diademata*, *Protocruzia adherens*, *Trochilioides recta*, *Suctoria* sp.

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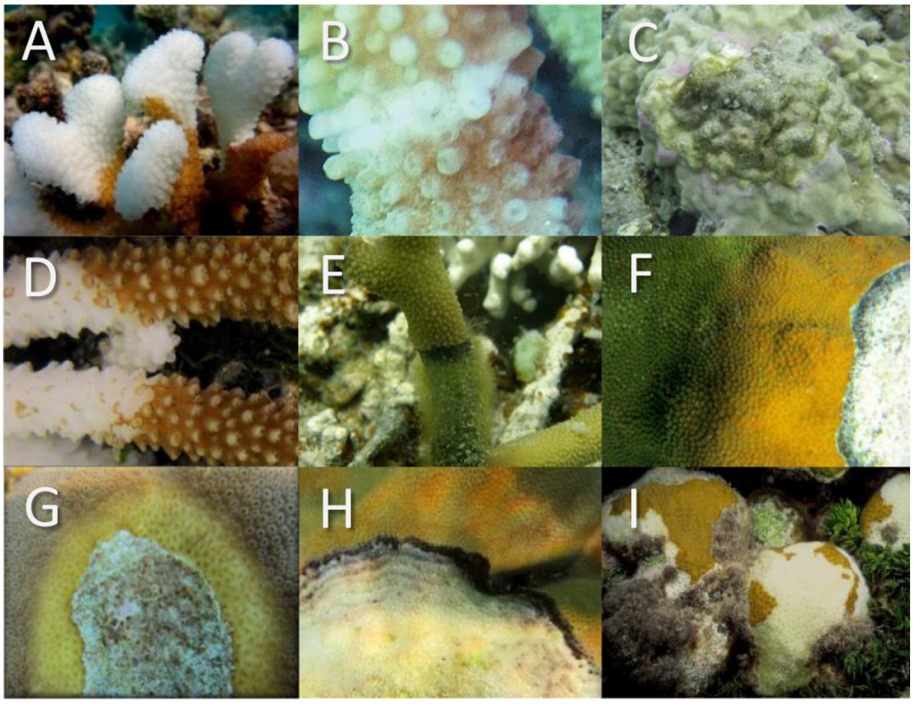

Fig. 1. Representative images of the diseases sampled in this study. A) White Syndrome, a sharp demarcation of apparently healthy tissue adjacent to the bare calcium carbonate skeleton, very prevalent in *Acroporas* and *Pocilloporas* throughout the Indo Pacific. B) Brown Band Disease, similar to that of WS but with the addition of a dark brown band visible beneath the advancing lesion interface, known to be caused by the ciliate *Philaster guamense*. C) *Porites* White Patch Syndrome, characterized by diffuse, medium to large, circular to oblong tissue loss, surrounded by a variable zone of swollen, paler tissues. D) White Band Disease, again similar to WS but currently only shown to affect *Acroporids* in the Caribbean. This disease is split into two main types, Types I and II. Type II differs in that the tissue adjacent to the exposed skeleton bleaches before dying. E) Skeletal Eroding Band, characterized by diffuse areas of tissue loss, exposing skeleton that is eroded and covered by the external casings (vacated loricae) of the ciliate *Halofolliculina*. F) Caribbean Ciliate Infection, similar to SEB but only affecting Caribbean corals. G) Caribbean Yellow Band Disease begins as pale, circular blotches of translucent tissue or as a narrow band of pale tissue at the colony margin, with affected areas being surrounded by normal, fully pigmented tissue. As the disease progresses, the tissue first affected in the center of the patch dies, and exposed skeleton is colonized by algae. H) Black Band Disease, characterized by a blackish band, 1 to 30 mm wide compromised of cyanobacteria. I) White Plague Disease is similar in appearance to WBD but affects different species. WPD is characterized by a sharp demarcation between apparently healthy tissue and the algal-colonized skeleton. Again, similar to WBD Type II, some cases have been shown to exhibit a narrow band of bleached tissue adjacent to exposed skeleton.

this randomness and the overall unevenness in the collection of samples associated with this study, we are unable to use the data for inferences in statistical analysis. Therefore we will only focus on describing the ciliate communities associated with each disease state without taking coral species and geographical location into effect.

2.2.2. PCR amplification of single cell isolates

After the morphological characteristics of the ciliates were described and photomicrographs taken, the individual ciliates were preserved in 100% absolute technical grade ethanol and stored at −20 °C until extraction and sequencing. DNA was extracted from the ethanol-fixed single isolates using a modified Chelex extraction (Walsh et al., 1991). At least 3 replicate isolates of the same ciliate species (identified by morphological characteristics above) were sequenced from the same geographical location, from the same coral species and from the same disease. All samples were vacuum centrifuged for 10 min and washed twice in sterile water (Sigma-Aldrich W3500) with a 2 min centrifuge step (20,000 *g*) in between. Following the final wash, 50 μl of a 5% Chelex 100 solution (made in the same sterile water as above) and 15 μl of proteinase K (20 mg/ml) was added to the cell isolate. The samples were subsequently left in a water bath overnight at 54 °C. After incubation, they were vortexed for 20 s, boiled at 100 °C for 10 min, vortexed for a further 20 s and centrifuged at 16,000 *g* for 3 min. Then 30 μl of supernatant was taken off and put in a fresh micro-centrifuge tube. This was then stored at −20 °C until further use. 20 μl PCR reactions (final PCR buffer contained: 1 mM $MgCl_2$, 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 extracted as above) were run in a Hybaid PCR-Express thermal cycler. The universal 18S rRNA gene eukaryotic primers 4617f (5′-TCCTGCCAGTAGTCATATGC-3′) and 4618r (5′-GATCCTTCTGCAGGTTCACCTAC-3′) were used following the PCR protocol of Oldach et al. (2000). The nested PCR reaction was carried out using 1 μl of a 1:100 dilution of the first round PCR product with the ciliate-specific primers 384f-cil (5′-YTBGATGGTAGTGTATTGGA-3′) and 1147r-cil (5′-GACGGTATCTRATCGTCTTT-3′), amplification conditions followed that of Dopheide et al. (2008). All sequences were ethanol-purified from PCR products and sequenced as per Sweet and Bythell (2012). Sequences of replicate ciliate samples were aligned using the Geneious 5.5.1 Beta version software. Only sequences which showed matches between replicates were classed as true reads and matched with sequences previously submitted on GenBank.

This combined approach of morphological and molecular profiling, allowed us to confirm our identification via morphology with the sequence data. In some cases identification was made to species level by matching the specific isolate morphologically identified to the retrieved sequence. In many more instances, however, the sequence data was less than a 98% match to ciliate sequencing in GenBank; therefore, identification could only be made to Genus level in these instances. A phylogenetic tree was constructed to aid further classification of the sequenced ciliates.

3. Results

There were no ciliates detected in apparently healthy coral tissue samples throughout, either by microscopic examination or molecular analysis, a result reflected by recent studies (Sweet and Bythell, 2012; Sweet and Bythell, 2015; Sweet et al., 2014). However, ciliates were found associated with all coral disease lesions assessed, consisting of

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Table 2
Morphological characteristics of the ciliates associated with coral diseases and light micrograph photo ID. Scale bars in photographs equal 10 μm.

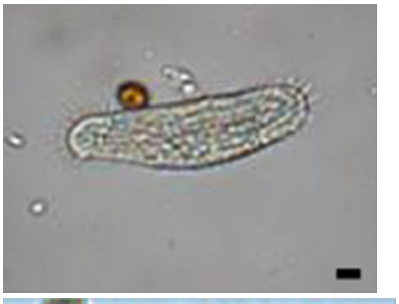
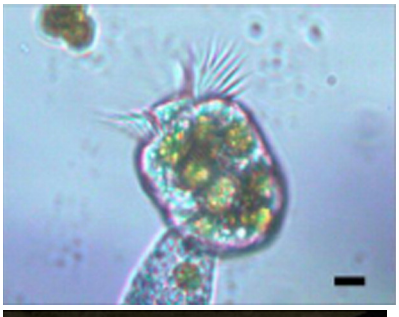
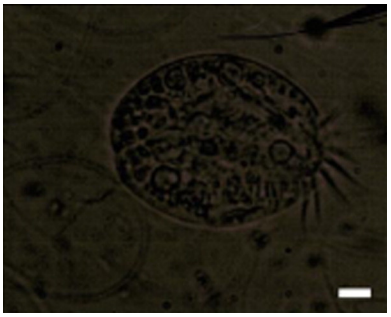
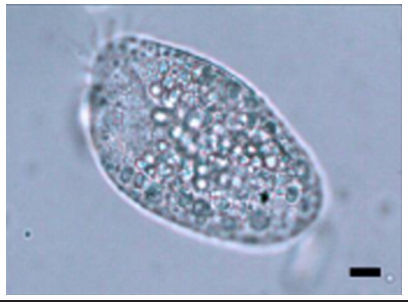
Species ID	Morphological description	Photo (scale bar 50 μm)
Diophrys sp.	Size: ca. 70 × 25 μm in vivo Shape: oval to slender oval with both anterior and posterior ends of the body more or less pointed. Color: ranges from gray to slightly yellowish. Structure: long ciliary organelles sometimes conspicuously, especially the caudal cirri and the anterior adoral membranelles. Length of buccal field 40–50% of body. Ciliature typical of the scutum-mode: 5 frontal, 2 pretransverse ventral, 5 transverse and 3 caudal cirri. Two marginal cirri conspicuously separated from each other, posterior one always being below transverse cirri. There are three large genus-typical caudal cirri present. Behavior: spins erratically around in circles, often seen to crawl along the coral skeleton	
Holosticha diademata	Size: ca. 80–90 × 25–50 μm in vivo. Shape: generally fusiform with both ends slightly narrowed and dorsoventrally flattened. Color: colorless Structure: cortical granules are prominent, blood-cell-shaped and sparsely distributed. Three frontal, one buccal, two frontoterminal, and 6–10 transverse cirri are often able to be observed under light microscopy. One contractile vacuole is present, post-equatorially located and not easily observed but varying contrast in your image can sometimes highlight it. Behavior: mainly crawling slowly on coral.	
Varistrombidium kielum	Size: ca. 55–75 × 40–50 μm in vivo. Shape: slightly asymmetric and has an elongated barrel-shaped posterior end usually with a blunt point. The collar region is clearly domed to form a conspicuous apical protrusion. Color: colorless with the presence of symbiotic algae (green to yellow) Structure: buccal cavity is shallow and inconspicuous and therefore unlikely to be seen in most light micrographs. The extrusomes are prominent, acicular, ca. 10 μm long, and evenly arranged alongside the dorsal side of cell and on narrowed upper equatorial and caudal areas. Macronucleus ovoid to ellipsoidal and members of this genus are often lacking micronuclei. Behavior: present on the coral skeleton, slow moving, crawling around	
Protocruzia adherens	Size: previously reported to range from ovoid to elongate but in this particular instance the ciliate's body was 65 μm long, 48 μm wide. Shape: asymmetric, ovoid ciliate with conspicuous cilia Color: colorless Structure: dorsal surface has reduced somatic ciliation which is often not visible. Buccal ciliation is massively developed in members of this Genus and they have a single macronucleus. The peristome begins at the pointed anterior end, and runs 1/3 of the body length. Behavior: present on the coral skeleton, slow moving, crawling around	
Trochilioides recta	Size: ca. 60 μm × 40 μm Shape: approximately oval, dorsal surface more strongly arched than the ventral surface Color: colorless Structure: ventral surface bears a posterior cytoplasmic spine. The spine has a specific gland attached to the end which produces an adhesive secretion for fixation to a substratum. Somatic ciliation is very much reduced in members of this genus and located only on the ventral surface. There are 2 contractile vacuoles visible within. Behavior: *T. recta* is often attached to the exposed coral skeleton.	

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Table 2 (*continued*)

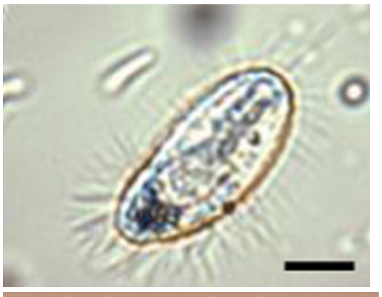
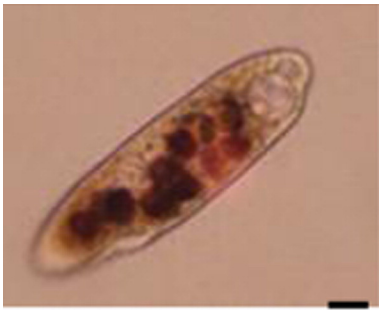
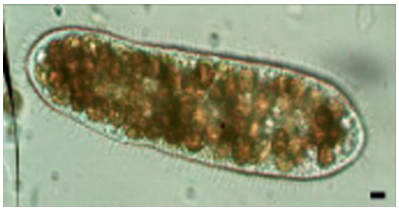
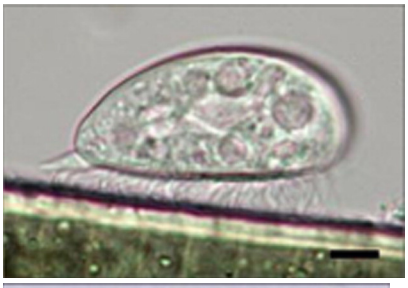
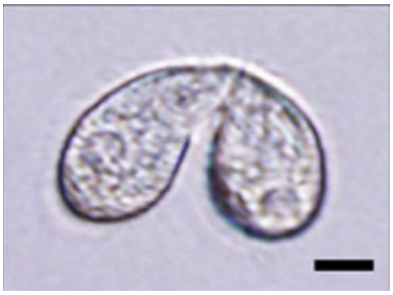
Species ID	Morphological description	Photo (scale bar 50 μm)
Uronema heteromarinum	Size: ca. 30 × 10 μm Shape: The body is long and ovoid Color: colorless Structure: truncated apical frontal plate and smooth pellicle. The cytostome is equatorially located with 12–14 somatic kineties visible. There is a single contractile vacuole caudally positioned with the pore near the posterior end. The cilia are arranged in about 15 longitudinal rows. The buccal cavity, which is situated anteriorly is small (1/6th of the body length) and characteristically triangular. A spherical macronucleus can be located in the region of the mouth and a contractile vacuole can be located at the posterior end of the cell. Behavior: fast moving around the exposed coral skeleton	
Philaster lucinda	Size: The body of this ciliate is slender but variable in size ranging from 60 to 200 × 20–60 μm in vivo. Shape: variable in outline from cylindrical to fusiform. The body is anteriorly narrowed and conspicuously pointed. Color: yellow to brown in color largely due to the endosymbionts being digested. Structure: length of buccal field is large, approximately 40–50% of body. The cytostome is conspicuous and deeply sunk. The macronucleus is band-like, twisted and positioned centrally with several micronuclei attached to it. One small, terminally located contractile vacuole is present and often visible. The cilia ca. 5–10 μm long; with oral cilia ca. 10–15 μm long and caudal cilium 12–15 μm in length. The paroral membrane is L-shaped, on the right hand side of oral cavity. Behavior: Locomotion on corals is characterized by fast, spiral swimming while rotating irregularly about its main body axis, motionless for short periods when feeding, often seen at the lesion interface or burrowing underneath the tissue and erupting from the mouth of individual polyps.	
Philaster guamense	Size: Body larger than *P. lucinda*, approximately 200–500 × 20–75 μm. Shape: Similar to many members of the genus, the outline is variable, cylindrical to fusiform; and anteriorly rounded. Color: The ciliates are colorless to brownish yellow, often with numerous food vacuoles or zooxanthellae. Structure: oral depression was conspicuous and deeply invaginated. The buccal field is ca. 30–40% of the cell length. The cytostome is clearly delineated by fibers, leading to a cytopharynx extending ca. 30% of the cell length. The macronucleus is sausage-like, elongate but often bent, positioned centrally along the main cell axis. Micronuclei were not observed, as prey (coral zooxanthellae and nematocysts) nuclei obscured identification. Behavior: seen to ingest coral tissue, less active than *P. lucinda* and appeared to play a secondary role in tissue digestion. Often seen hanging back from the main lesion advance, congregating in large numbers giving the appearance of the brown band in BrB disease. These ciliates often attach themselves by one end and hang horizontal and perpendicular to the skeleton. Division of the ciliate was rarely noted in preserved specimens but commonly seen and often resulted in a ciliate similar to appearance to *P. lucinda*.	
Trochilia petrani	Size: ca. 40 μm × 20 μm Shape: The body of this ciliate is approximately oval with dorsal surface strongly arched and often longitudinally ribbed, with the ventral surface flattened. Color: colorless Structure: presence of a posterior cytoplasmic spine from which a thread may be secreted for attachment purposes, similar to *Trochilioides recta*. Somatic ciliation severely reduced and located only on the ventral surface. The macronucleus is large, ovoid and of the heteromeric type. There are 2 contractile vacuoles. Behavior: crawls across the skeleton, slow moving	
Glauconema trihymene	Size: Body size ranges from 30 to 36 × 16–24 μm in vivo. Shape: The ciliate has a trophont and tomite stage. It is bilaterally flattened with a large apical plate in the trophont and long, oval to fusiform with a small apical plate in the tomite. Color: colorless Structure: buccal cavity is spacious in the trophont while narrow in the tomite. Both have a single spherical macronucleus. The contractile vacuole is positioned caudally. There are 17 somatic kineties, with cilia ca. 8–10 μm long. Behavior: locomotion by crawling slowly with frequent pauses in case of trophont or swimming quickly in case of tomite.	

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Table 2 (*continued*)

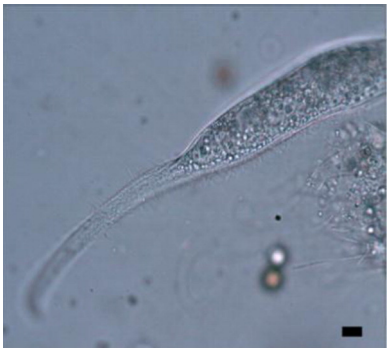
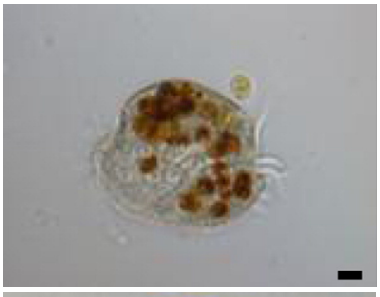
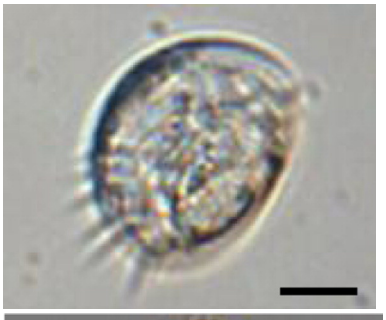
Species ID	Morphological description	Photo (scale bar 50 μm)
Litonotus pictus	Size: body size ranges considerably from 150 to 280 mm in vivo Shape: body is laterally compressed, highly elongate with anterior neck-like region which bends towards the dorsal edge. Color: colorless Structure: oral aperture slit, on convex edge of neck extending less than halfway down the body with two elongate macronuclear nodules. The macronucleus is commonly in two spherical parts with a single micronucleus wedged between the two. There is a single, bar shaped contractile vacuole subterminally located running along the entire ventral margin. Ciliation is present on both the lateral surfaces with some longer cilia on the neck region forming a mane-like structure. There is often one to several contractile vacuoles present. Behavior: moves in a slug-like fashion, contracting and extending the body, appears to search for food with the neck-like region	
Euplotes sp.	Size: ca. 90–140 μm long in vivo Shape: body of this ciliate is slightly rectangular in outline, with no conspicuous dorsal ridges Color: central area (known as the cytoplasm hyaline) is often dark due to food vacuoles and granules Structure: length of buccal field is about 65% of body. The macronucleus is C-shaped and the micronucleus spherical. AZM distinct, with about 60 membranelles, proximal portion curved ca. 90° to right. Ten frontoventral cirri are in a genus-typical pattern; two left marginal cirri separated and aligned evenly with 3 or 4 caudal cirri. Behavior: locomotion by medium-fast crawling on coral, sometimes stationary for long periods.	
Euplotes sp.	Size: ca. 60–70 × 50–60 μm in vivo Shape: body is oval in outline and dorso-ventrally flattened. Color: colorless with symbiotic algae prevalent once ingested Structure: length of the buccal field stretches ca. 75% of the body. Similar to the ciliate above, the macronucleus is C-shaped and micronucleus spherical. Proximal portion of AZM curved at ca. 90° to right. Behavior: locomotion of this *Euplotes* is by slow, slightly jerky, crawling on coral, remaining stationary for long periods.	
Aspidisca sp.	Size: round 25 × 25 μm Shape: They are small, with irregularly ovoid, inflexible bodies with well defined dorsal ridges. Color: colorless Structure: AZM cannot be visibly seen under light microscopy. Interestingly, the AZM is currently thought to be rudimentary in other described species from this genus. The cirri are arranged in two groups; a transverse set of 5 or more large cirri located posteriorly and an anterior group (often 7). The macronucleus is horseshoe-shaped or often separated into two parts. The contractile vacuole is positioned posterior to the transverse cirri. Behavior: ciliates are known to be euplotine hypotrichs, free-living and often sapropelic.	
Pseudokeronopsis sp.	Size: large ciliate often reaching in excess of 350 × 90 μm in vivo. Shape: body is long, elliptical with the anterior end broadly rounded (posterior end more narrowed) and dorsoventrally flattened Color: commonly, dark reddish in color. Structure: two types of cortical granules: 1, pigmented orange, mainly grouped around the cirri and dorsal bristles; and the 2nd is round and colorless, lying just beneath the first. There are ca. 9–13 pairs of frontal cirri arranged in two rows forming a bicorona. AZM is conspicuous with 68–92 membranelles and extends far onto right side of cell. One contractile vacuole is visible and positioned at the posterior end of the body. There are numerous macronuclear nodules present. Behavior: locomotion is by slow crawling. Other members of this genus have been shown to feeds on a variety of other protists.	

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Table 2 (*continued*)

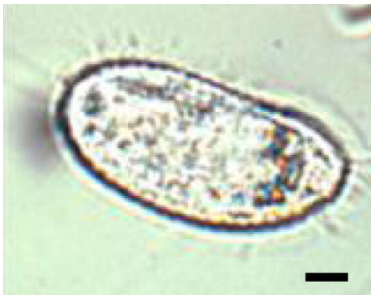
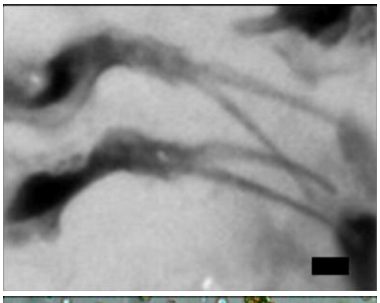
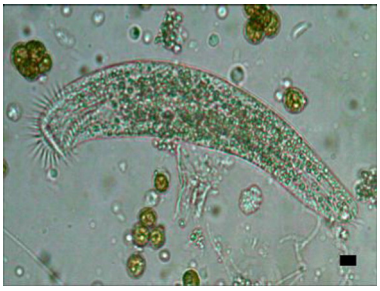
Species ID	Morphological description	Photo (scale bar 50 μm)
Hartmannula derouxi	Size: body ranges from 60 to 120 × 30–70 μm in vivo. Shape: long and oval to elongate in outline. Color: colorless Structure: pellicle is covered with a smooth, thin, colorless gel-like substance. There are many contractile vacuoles present in this species. The podite is ca. 20 μm long and secretes a glue-like substance for adhering to substratum. The macronucleus is ellipsoidal. Behavior: small erratic movements on the exposed skeleton, often fast when moving in a straight line	
Licnophora macfarlandi	Size: ca. 40–70 μm Shape: The body of this ciliate is squat and consists of two distinct regions (the oral and basal region). The neck is short, not clearly distinguishable from adjacent oral and basal regions. Basal region round and the surface is slightly concave. It is surrounded by a single circular ring of short, densely packed cilia of uniform length. Color: colorless Structure: The oral region fringed by a broad band of AZM before plunging into the infundibulum. The rows of membranelles are separated by sharply pointed endoplasmic ribs. First two thirds of AZM follows periphery of body, last third of AZM deviates from body periphery spiraling inward towards center of oral region. The center of the aboral surface is smooth without cilia, fringed by AZM. Behavior: Previously only found associated with echinoderms, attached to skeleton and doesn't move.	
Halofolliculina coralliasia	Size: ca. 80–120 μm long × 20–30 μm in width Shape: ciliate has a large and elongated body, especially in the neck region, with a conspicuous pair of peristomial wings extending from the lorica (a structure generally cylindrical, with a definite bend). Color: body is highly contractile and often pigmented but can be clear. Structure: lorica bears the prominent buccal ciliature; somatic ciliation uniform and holotrichous. Behavior: embedded in the coral skeleton, also seen in less dense numbers on the apparently healthy tissue in advance of the lesion	
Anteholosticha sp.	Size: ca. 90–125 × 30–45 μm in vivo. Shape: slender to ellipsoidal in outline Color: colorless with yellow-greenish cortical granules Structure: single contractile vacuole positioned on the left around the mid-body. There are ca. 117 macronuclear nodules; and three to four micronuclei are present in members of this genus (however these can only be observed during morphogenesis). Behavior: observed randomly moving around the exposed coral skeleton	

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Table 2 (*continued*)

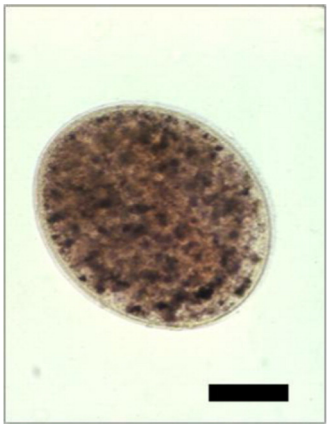
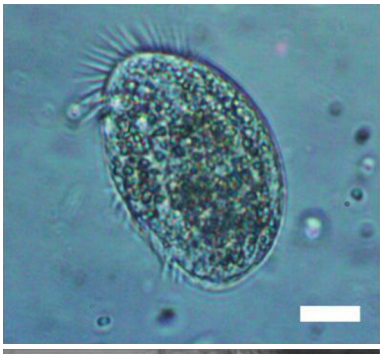
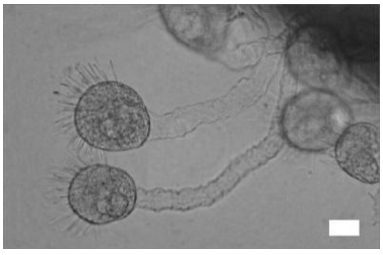
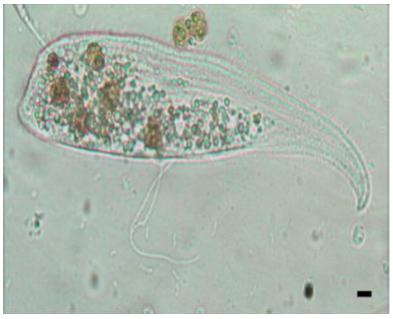
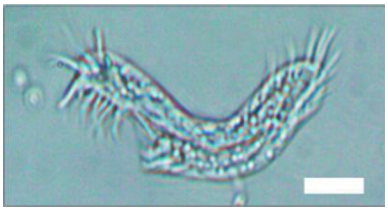
Species ID	Morphological description	Photo (scale bar 50 μm)
Cryptocaryon sp.	Size: ca. 20–30 × 50–70 μm Shape: oval to pear-shaped Color: dark brown to black Structure: simple buccal apparatus consisting of cytosome surrounded by a ring of cirri-like structures. Lacks a distinct buccal cavity and oral membranelles distinct in other members of this order Behavior: complex lifecycles with five stages. The trophont (the feeding stage) is the stage observed embedded within host tissues such as many species of fish. After the trophont stage the ciliate becomes a protomont which then encysts and transforms into the tomont (the reproductive stage). The tomont develops and divides into numerous tomites, which eventually leave the cyst as theronts, the free-swimming infective stages (which was the stage seen associated with corals) are very motile.	
Dysteria derouxi	Size: variable, ranging from 75 to 110 × 30–50 μm in vivo. Shape: bilaterally flattened and when observed from the side, looks rectangular in outline, with the ventral side straight and dorsal slightly convex. The anterior margin is usually transverse truncate, and posterior region somewhat narrowed. Color: cytoplasm colorless to grayish, usually containing many food vacuoles (ca. 5 μm in diameter) and several ingested diatoms ca. 10–30 μm long. Structure: right plate arched and slightly wider than the flattened left plate. Ciliary rows restricted to the anterio-ventral groove between the two plates. The podite is ca. 12 μm in length, the distal end can be pointed or blunt and emerges from the left posterior ventral side. There are two ventral contractile vacuoles, 6–12 μm in diameter. The macronucleus is elongate, conspicuously large, centrally positioned and characteristically heteromerous. No micronucleus detected. Cilia on average are ca. 10 μm long. Behavior: movement moderately rapid, usually "standing" on coral skeleton with podite slowly extending back and forth while the ciliate 'crawls' forward.	
Paracineta limbata	Size: ca. 100–150 μm × 10–20 μm Shape: generally globose, oval or obconic and branched Color: colorless Structure: lack cilia and other locomotor organelles in their final stage of their lifecycle. They have a pair of tentacles for capturing food but lack a mouth. Each tentacle has an outer contractile sheath, with myonemes and an inner tube from the ectoplasm. The body is spherical to ellipsoid in shape, 20–50 μm in diameter with much of it protruding out from a cup-shaped lorica. The lorica may be elongated posteriorly but it always terminates in a true stalk. Tentacles arise and radiate out from that entire portion of the body that protrudes from the lorica. Behavior: always attached to the exposed skeleton.	
Trachelotractus sp.	Size: A large ciliate, 1300 μm × 35 μm. Shape: vermiform with a conspicuous, globular head which is distinctly set off from narrowed, grayish neck/trunk and gradually narrowed at the posterior end (but not tail-like). Color: colorless cytoplasm, containing many 1–5 μm sized fat globules, innumerable 3 × 2 μm sized ellipsoid inclusions, and many small (7–20 μm long) diatoms Structure: macronuclei are small, globular to slightly ellipsoidal and distributed throughout body. The contractile vacuole is distinct, located in the posterior with a single excretory pore in the center. Long extrusomes which are usually curved and/or wrinkled and are attached to the peribuccal ridge gives the ciliate a conspicuous apical beard (if completely extruded). The cortex is conspicuous, forming a 2 μm thick, vitreous layer which is sharply separated from the granular cytoplasm. Behavior: very flexible and contractile, but rather slow moving. Whether the diatoms seen inside the ciliate were actively ingested or contained in prey organisms has not been observed.	
Hemigastrostyla enigmatica	Size: ca. 15–20 μm × 60–100 μm Shape: elongate with both ends widely rounded, with the frontal portion head-like. Dorsoventrally, the ciliate is significantly flattened. The dorsal side is uneven, with the central part thicker than the posterior and anterior ends. Color: colorless, with food vacuoles containing mostly flagellates and diatoms. Structure: pellicle, cortical granules are very fine (<1 μm), generally grouped into rosette-like and close to cilia. Two large macronuclear nodules, ellipsoid in shape are located in the mid-body. Characteristically 2 right marginal cirri arising from the body can be seen to slowly wave up and down, while all the other ciliary organelles are stationary. The AZM is about half of the ciliates length with distal end of zone extending back far onto right side. Behavior: movement is slow to moderate, often crawling on the coral skeleton. They can however swim, slowly rotating around its longitudinal axis.	

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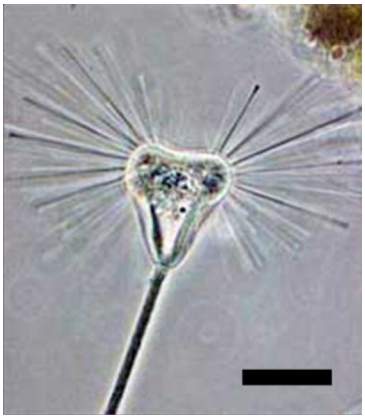
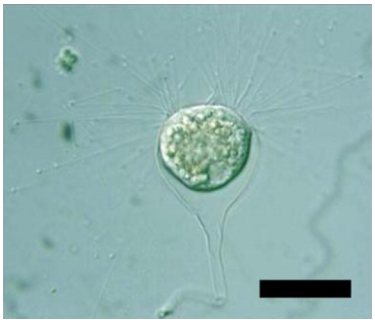
Table 2 (*continued*)

Species ID	Morphological description	Photo (scale bar 50 μm)
Chaenea vorax	Size: ca. 15–23 μm × 70–100 μm Shape: elongate, rounded and highly contractile. The posterior may be rounded or with a narrowing tail region. Color: colorless, sometimes brown green food items present in food vacuoles Structure: anterior is always with a short, non-extensible snout-like region which bears many more closely packed cilia than the rest of the body. The oral aperture is oval and located apically. The whole body is covered in uniform longitudinally arranged ciliary rows which spiral slightly. Trichocysts are often common, particularly in the snout region. The macronucleus is often found in one or more rounded parts and there is always a large terminal contractile vacuole present. Behavior: known to be a predatory marine gymnostome.	
Acineta sp.	Size: stalk 40–60 μm in length, body ca. 10–15 μm × 10–15 μm Shape: inverted conical shape within a cup-like lorica. The body completely fills the lorica which is flattened and borne upon a stalk. The lorica opens apically, as a dump-bell shaped slit. Color: colorless Structure: body and tentacles protrude through this aperture. The tentacles which are commonly capitate are arranged in 2 to 3 distinct fascicles. The macronucleus is rounded to ovoid. Behavior: stationary, attached to the coral skeleton with the stalk	
Suctoria sp.	Size: stalk 40–60 μm in length, body ca. 10–15 × 10–15 μm Shape: The body is spherical and extended posteriorly as a small cone form which a fine stalk arises. Color: colorless with greenish food vacuole in body structure Structure: a few tentacles arise from the body and they have distinctly thickened ends. There are 2 contractile vacuole and a macronucleus which is spherical. Behavior: stationary, attached to the coral skeleton with the stalk	
Condylostoma sp.	Size: ca. 40–50 μm × 100–150 μm Shape: elongate and ovoid. Color: colorless with symbiotic algae in the food vacuoles Structure: anterior end is truncated and carries a conspicuous AZM on the left and a large undulating membrane on the right of an unciliated, wide V-shaped peristome. The body is uniformly ciliated and a single terminal contractile vacuole with forward leading serving canals can be observed. The macronucleus is moniliform and the ciliate has a characteristic triangular peristome with a large undulating membrane. Behavior: active around the lesion interface, appeared to be eating expelled symbiotic algae from the coral	

28 different ciliate species (Tables 1 and 2). We have split the results into different coral diseases for ease of understanding (see below).

3.1. White Syndrome

Three different species of coral (*A. muricata*, *A. aspera* and *P. damicornis*) showing signs of WS (Fig. 1A) were examined from five different locations, Australia, Solomon Islands, Fiji, Maldives and Aquaria within the UK. In total twenty different ciliate species were identified from the WS lesions across this geographical range (Tables 1 and 2). Although, there was slight variation between locations, three ciliate species; *Philaster lucinda*, *Holosticha diademata* and a *Pseudokeronopsis* were detected in all samples.

3.2. Brown Band Disease

Only four fragments of the coral *A. muricata* showing signs of BrB (Fig. 1B) were sampled and only from one location; Heron Island in Australia. Eight species were identified from these corals showing signs of BrB (Tables 1 and 2). *P. guamense* (Table 2), was the dominant

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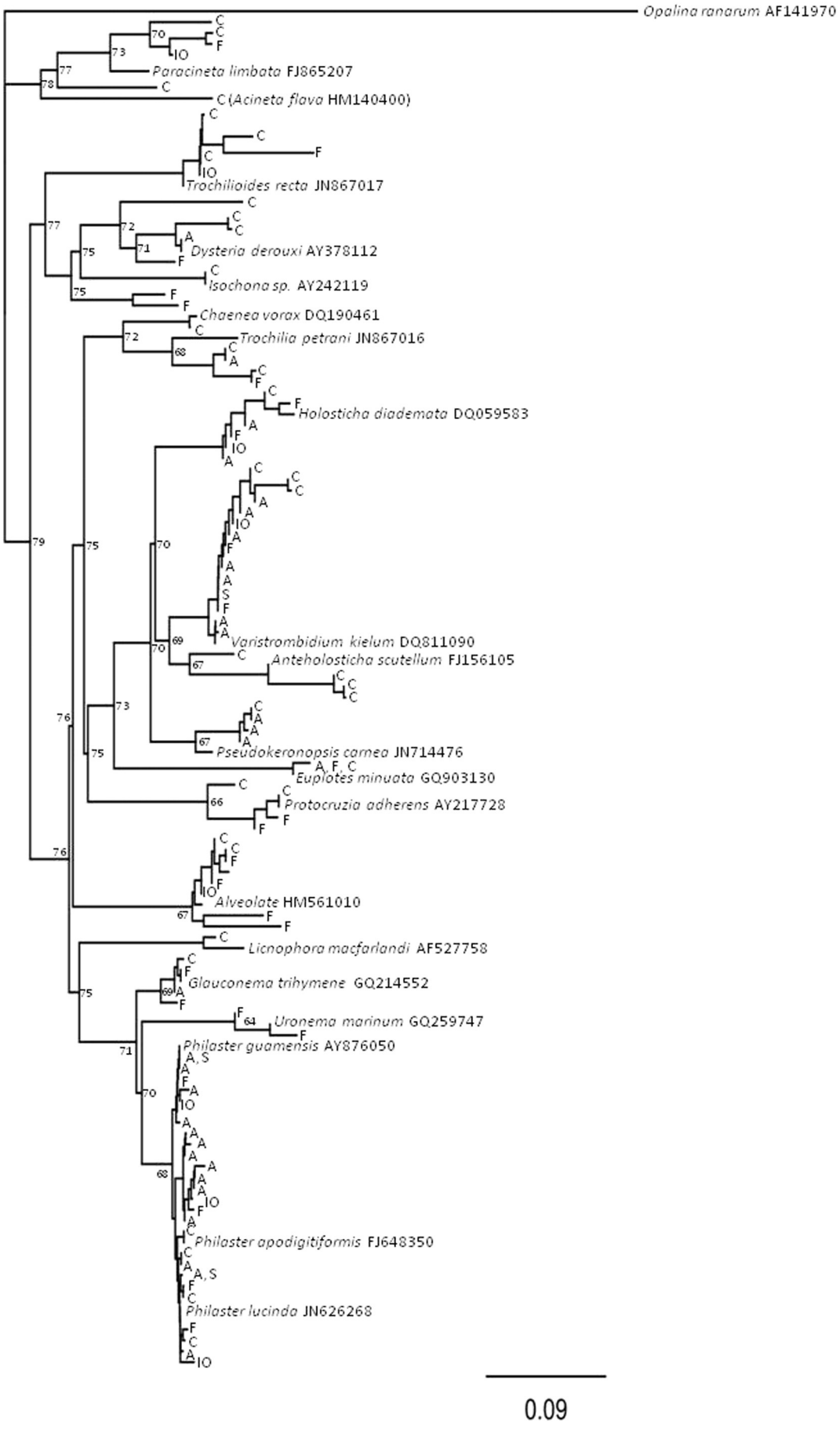

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ciliate in all BrB samples. However, there was also an abundance of *P. lucinda*, the dominant ciliate associated with WS. In addition to all the BrB samples, *P. guamense* was only detected in one other disease, WS, yet not in all replicates of this disease (Table 1).

3.3. Porites White Patch Syndrome

Nine colonies of *P. lutea* showing signs of PWPS (Fig. 1C) were sampled from three locations, Moyonette, Reunion and South Africa. Only six species of ciliate were identified from PWPS and there was no variation in the ciliates observed between locations. Five of the six species, *H. diademata*, *Varistrombidium kielum*, *Uronema heteromarinum*, *Philaster lucinda* and *Dysteria derouxi* were also detected in WS samples. Only one species, *Paracineta limbata* (Table 2) was present on this disease yet absent in both WS and BrB.

3.4. White Band Disease

WBD (Fig. 1D) had the third most diverse community of ciliates associated with any coral disease (Table 1). *A. cervicornis* from two different locations, Venezuela and Columbia were studied. The ciliate community was the same at both sites. Despite being from the Caribbean compared to the Indo-Pacific (for WS, BrB and PWPS, above) there were many similarities with the ciliate species identified. Species such as *V. kielum*, *Protocruzia adherens*, *Trochilioides recta*, *P. lucinda*, *Trochilia petrani*, *Glauconema trihymene*, *Pseudokeronopsis* sp., *Licnophora macfarlandi*, *D. derouxi*, *P. limbata*, and *Chaenea vorax* were present in corals showing signs of WBD, WS, BrB and PWPS. However, although the species ID were the same (both from GenBank sequence matches and morphological descriptions), there were certain mis-matches in the sequence data, showing there may be slight geographical variation between these species found in the Caribbean and those in the Indo-Pacific (Fig. 2). Interestingly, only two species, an unidentified *Anteholosticha* sp. (Table 2) and a *Trachelotractus* sp. (Table 2) were detected in samples with WBD and not previously identified in other diseases.

3.5. Skeletal Eroding Band

Although, we only had a small sample size for this disease (Fig. 1E) and samples were only from one coral species, *A. muricata*, there was still a diverse ciliate community associated with the lesion interface (see Table 1). Furthermore, the small sample set was separated over three countries, Moyonette, Reunion and South Africa, yet the ciliate diversity associated with the disease remained the same. There were ten species associated with SEB (Tables 1 and 2), however all samples were dominated by one main species, *H. corallasia* (Table 2). A result reflected in previous studies (Page et al., 2008). One further species which was only identified associated with SEB was *Condylostoma* sp. (Table 2), potentially indicating another previously un-described disease specific ciliate.

3.6. Caribbean Ciliate Infection

Similar to SEB, CCI (Fig. 1F) was dominated by *H. corallasia* (Table 2), morphologically similar to that associated with SEB and a 100% GenBank match in DNA sequence. However, in contrast to the diverse ciliate community associated with SEB, CCI was only associated with two other ciliate species; *P. lucinda* and a *Suctoria* sp. (Table 2). Interestingly, sequences from *P. lucinda* (a total of n = 36 isolates equally split between locations), found in both the Caribbean and the Indo-Pacific showed slight mis-matches (1–2 bp) over the total sequence read length (see Fig. 2 for phylogenetic spread of a subset of these sequences). Interestingly, CCI showed the lowest number of ciliates compared to all other diseases assessed (Table 1). However, similar to SEB, only four corals showing this disease sign were available for sampling, all of which came from only one coral species, *A. cervicornis*, and from one geographical location Venezuela.

3.7. Caribbean Yellow Band Disease

Although only one geographic location was sampled for YBD (Fig. 1G), fragments were taken from eight different colonies of *O. annularis*. YBD showed the second lowest diversity of ciliates associated with any coral disease lesion, with only four ciliate species being recorded in total. However, these four were present on all replicate samples of the disease, suggesting a stable community. Interestingly, this was the only coral disease assessed in this study which did not show the presence of *P. lucinda* (Table 1). However, the low ciliate diversity found in both CCI and YBD may be a factor derived from the low sample size and further study should be conducted on these diseases.

3.8. Black Band Disease

Corals showing signs of BBD (Fig. 1H) were sampled from five different locations (Table 1) and from seven coral species (two *Diploria* species, *O. annularis*, *C. natans*, *A. muricata*, *P. lutea* and *G. djiboutiensis*) (Table 1). There appeared to be no variation in ciliate diversity between species or between location. Furthermore, despite the larger sample size for this disease than in many other cases, the ciliate diversity was consistently low, with only five species identified throughout all the samples.

3.9. White Plague

Equal samples sizes (n = 8) of corals showing signs of WP (Fig. 1I) as those for WB were used for analysis, however in this case two different coral species *C. natans* and *O. annularis* were sampled. 14 ciliate species were recorded from these diseased corals. Interestingly, similar to the results shown for WB and WS, slight variations (1–2 bp) in the ciliate sequences retrieved were also observed, suggesting slight geographic variation may be occurring between the ciliates residing in the Indo-Pacific and those present in the Caribbean (Fig. 2).

For a detailed description of the morphology and behavior of all the ciliate species identified above see Table 2.

4. Discussion

Protozoa are a dominant group of microorganisms, likely found in all environments, however they are a relatively understudied group and little is known about species distribution. Regarding corals specifically, only a handful of ciliate species have currently been described and all have been shown to be associated with disease lesions (Page et al., 2008, Sweet and Bythell, 2012; Katz et al., 2014; Sweet et al., 2014; Randall et al., 2014). BrB (Bourne et al., 2008; Randall et al., 2014), SEB (Page et al., 2008), CCI (Croquer and Weil, 2009), WPD, BJS (Randall et al., 2014), WS (Sweet and Bythell, 2012) and WBD (Sweet et al., 2014) have all been shown to have some association with specific ciliate causal agents. However, except for the latter two studies, molecular analysis was not conducted to assess the complete diversity of ciliates associated with the lesion interface. Here we show that all diseases studied (nine different pathologies) showed the presence of a ciliate community. However, due to the randomness in collection of the samples we were unable to assess, at least statistically, any variation

Fig. 2. Neighbor-joining consensus tree of partial 18S rRNA gene sequences of species of ciliates found within coral diseases in Australia (A), Fiji (F), the Solomon Islands (S), the Indian Ocean (IO) and the Caribbean (C). 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 neighbor-joining consensus tree (1000¥ re-sampling) was constructed in Geneious Pro 5.0 using the Tamura genetic distance model with an opalinid protist, *Opalina ranarium* (AF141970), as the outgroup.

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which occurred between the different coral species, disease states and/or geographical location. Furthermore, we were also unable to assess any temporal variation which may have occurred, as only one sample collection period was conducted at each location.

The majority of the ciliates identified in this study such as *H. diademata*, *P. adherens*, *Trochiloides recta* and *U. heteromarinum* have already been previously described as bacterivores in other ecosystems (Doherty et al., 2010). Therefore they are likely colonizers of the exposed coral skeleton left by the advancing disease lesion and/or feeding off the bacteria associated with the dead and dying tissues of the coral. In contrast, two other species, the *Pseudokeronopsis* and the *L. macfarlandi* are known to be ciliativores and these are therefore likely attracted to the diseased coral with the goal to feed on the other associated ciliate community members. *L. macfarlandi* for example, has previously been described as an eco-parasite which attaches itself to the inner wall of the respiratory tree of sea cucumbers (Long et al., 2006). Having found this species on corals, it may be likely that *L. macfarlandi* is a generalist and will be found associated with numerous different marine organisms in the future.

Although, many of the species of ciliates detected on the lesion interfaces of corals are likely secondary invaders (as stated above), a few have been observed burrowing into and underneath coral tissue in advance of lesions (Sweet and Bythell, 2012; Sweet et al., 2014). Furthermore, out of the 28 species associated with the different diseases studied, six showed the presence of symbiotic algae internally. These included; the *V. kielum*, *P. lucinda*, *P. guamense*, a *Euplotes* sp., a *Trachelotractus* sp. and a *Condylostoma* sp. Although, there is no evidence available to confirm if these algae are from the coral themselves or a symbiotic relationship with the ciliates, the two *Philaster* species in particular warrant further investigation with regard to their possible roles in coral diseases. For example, Katz et al. (2014) recently induced the signs of BrB on otherwise healthy corals by placing *P. guamense* on mechanical scars (either experimentally wounded or those caused by the Crown of Thorn Starfish). Indeed, other members of this group, the Scuticociliata, have been shown to be pathogenic to a wide variety of different organisms (Bradbury et al., 1996; Song and Wang, 1993). These include everything from dolphins to whales (Poynton et al., 2001; Sniezek et al., 1995; Song et al., 2009), clams to flounders (Cremonte and Figueras, 2004; Kim et al., 2004) and even seahorses (Cheung et al., 2006). In fact, 'Scuticociliatosis', is the name given to diseases caused by these opportunistic histophagus parasites and is routinely characterized by the ciliates' high potential for systemically invading hosts, which often results in high mortalities (Jee et al., 2001).

In conclusion, this is the first study to highlight a diverse community of ciliates associated with all nine of the coral diseases studied here. There is no doubt that the majority of these species are opportunistic scavengers, simply feeding off the bacteria associated with the exposed skeleton and/or the bacteria associated with the necrotic tissue, however a few warrant further investigation with regard to their specific roles in disease pathology worldwide.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.seares.2015.06.008.

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