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Identification of a Ciliate (Oligohymenophorea: Scuticociliatia) Associated with Brown Band Disease on Corals of the Great Barrier Reef[▽]

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A ciliate associated with the coral disease brown band (BrB) was identified as a new species belonging to the class Oligohymenophorea, subclass Scuticociliatia. The ciliates were characterized by the presence of large numbers of intracellular dinoflagellates and displayed an elongated, tube-shaped body structure. They had uniform ciliature, except for three distinct cilia in the caudal region, and were typically 200 to 400 μm in length and 20 to 50 μm in width.

Coral reef ecosystems have been exposed to increasing levels of sedimentation, nutrient enrichment, and ocean warming in the past few decades (1, 20–22), resulting in corals experiencing elevated levels of stress and enhanced susceptibility to disease infection (9, 19, 20, 23, 31). Coral disease epizootics have become a major threat to reef ecosystems globally, with reports of newly emerging syndromes continuing to increase in numbers (17, 41). Identifying the microbial communities associated with coral diseases is critical to further current understanding of how environmental and climate changes might affect the prevalence of diseases. To date, a wide range of microorganisms, including fungi, bacteria, and cyanobacteria, have been identified in association with both healthy and diseased corals (10, 14, 18, 29, 30, 32, 33, 44), although microbial communities associated with many coral diseases remain unknown (41).

Seven coral diseases on the Great Barrier Reef (GBR) have been described previously (42), although their causative agents remain largely undescribed. One disease, named brown band (BrB), was described for the first time in studies of corals in three families (Acroporidae, Pocilloporidae, and Faviidae) in the northern and southern sectors of the GBR (42). Macroscopic symptoms of the disease manifest as a brown zone, which is preceded by healthy tissue and followed by exposed white skeleton as it progresses across the coral (see Fig. 1a). In some cases, a white zone, comprising bleached tissue and/or denuded skeleton, is observed between the brown band and healthy tissue. The distinctive brown color that constitutes the macroscopic field signs of BrB is derived from a mass of unknown ciliates gliding over the exterior surface of coral samples and into the coelenteron and cavities of the coral polyps. Here we report the identification of the ciliate associated with BrB by use of microscopic and molecular approaches.

Ciliates were removed from specimens of the staghorn coral (*Acropora muricata*) exhibiting signs of BrB. Disease samples were collected from Davies Reef ($n = 3$) located in the central sector of the GBR (18°49.86′S, 147°38.2′E) and from fringing reefs around Heron Island ($n = 1$) located in the southern sector of the GBR (23°44.17′S, 151°91.25′E). All samples were taken from near the advancing front of the disease lesion and encompassed the brown band ciliate mass. Although potentially a complex microbial community involving bacteria, diatoms, dinoflagellates, and other microscopic marine plankton, the ciliate population appeared uniform and dominated by one morphologically distinct protozoan (Fig. 1b). Ciliates removed from coral specimens were processed for microscopic analysis by fixation in Bouin's solution (13, 15) and stored in the dark at 4°C or kept at −80°C until DNA was extracted.

High densities of intracellular zooxanthellae (*Symbiodinium* sp.) were observed within all ciliates examined by use of light microscopy (Fig. 1c). Morphologically, the ciliate had an elongated, tube-like shape rounded at both the posterior and apical ends (Fig. 1c). The length of the ciliate ranged from 200 to 400 μm, while the width ranged from 20 to 50 μm. Ciliation was uniform over the surface of the organism (Fig. 1c) except for three distinct and extended cilia in the caudal region. Scanning electron microscopy (SEM) revealed the oral apparatus to be differentiated from somatic ciliature and located in the buccal cavity on the ventral side (Fig. 1d).

Total DNA from ciliate and coral tissue samples (extracted according to the methods described in references 6 and 43) was amplified with conserved eukaryotic primers (18S-6-CIL-V and 18S-1511-CIL-R) (15). PCR resulted in amplification of the 18S rRNA genes from protozoa and other eukaryotic organisms (by PCR cycling performed at 95°C for 3 min followed by 30 cycles at 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min and a final extension step of 72°C for 7 min), with the products (~1.8 kb) cloned (TOPO TA cloning kit; Invitrogen) and the insert 18S rRNA gene reamplified from individual

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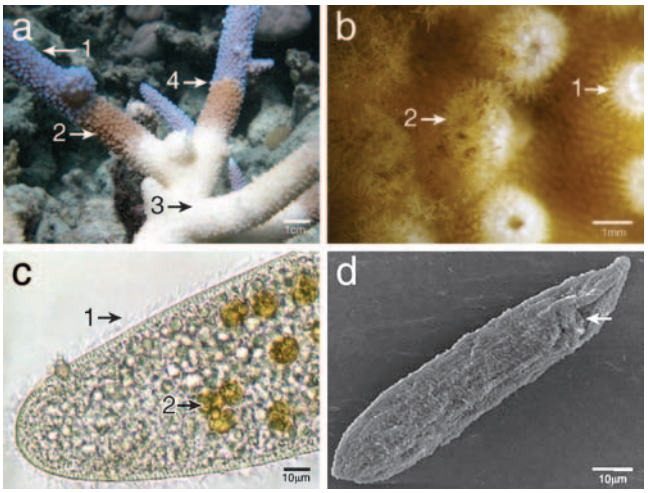

FIG. 1. (a) Photograph of brown band disease on a branch of *Acropora muricata* showing its macroscopic field signs. Arrow 1, healthy coral tissue; arrow 2, brown zone preceded by healthy tissue; arrow 3, exposed white skeleton following the brown zone; arrow 4, a white, bleached zone between the brown band and healthy tissue. (b) Micrograph showing a coral polyp covered by a mass of ciliates (Leica MZ16A; Leica Microsystems AG, Wetzlar, Germany). Arrows 1 and 2 are as defined for panel a. (c) Micrograph (obtained using an Olympus Vanox AH-2 compound microscope; Shinjuku-ku, Tokyo, Japan) (×100 magnification) showing morphology of the brown band ciliate. Arrow 1, uniform ciliation; arrow 2, *Symbiodinium* cells within a ciliate. (d) SEM micrograph (obtained using a JEOL JSM 5410LV scanning electron microscope) showing morphology of the brown band ciliate. The arrow denotes the buccal cavity on the ventral side. Dissecting and compound images were taken using an Olympus digital camera (C-5050Z) (5 megapixel, 3× zoom).

clones. Restriction fragment length polymorphism analysis was performed on reamplified products (8), and clones were grouped into operational taxonomic unit (OTU) groups. Clones sequenced from the dominant OTU groups were affiliated with *Symbiodinium* species within the clade C lineage (98% to 99% sequence identity). One OTU group was affiliated with 18S rRNA gene sequences of ciliates within the scuticociliate family and was putatively identified as derived from the dominant BrB ciliate organism. This sequence demonstrated 95% sequence identity (over 1,749 bp) to the 18S rRNA gene sequence of the *Parauronema longum* ciliate. Phylogenetic comparisons indicated that the unknown ciliate is related to other ciliates belonging to the class Oligohymenophorea, subclass Scuticociliatia (Fig. 2). This subclass includes the scuticociliates *Schizocaryum dogieli*, *Cohnilembus verminua*, *Anophyroides haemophila*, *Pseudocohnilembus marinus*, and *Uronema marinum*. Scuticociliates often feed on bacteria, using complex morphological adaptations to create currents and filters capable of capturing bacteria and other particles from the water column or scraping them from hard surfaces (25).

Based on the retrieved 18S rRNA gene sequence, new PCR primers were designed using the oligonucleotide primer algorithm of the ARB package (27). Generated primers were checked against the GenBank database by a standard nucleotide-nucleotide BLAST search (3). PCR primers specific for the identified BrB ciliate included BrB-F-171 and BrB-R-1721 (Table 1) (PCR cycling was performed at 95°C for 3 min followed by 35 cycles at 95°C for 30 s, 45°C for 45 s, and 72°C for 2 min, with a final extension of 72°C for 10 min). Further BrB tissue samples from both Davies Reef and Heron Island were amplified and clones screened as described previously. Sequencing of the dominant clone types retrieved almost identical ciliate-affiliated 18S rRNA gene sequences (>98% sequence identity) as obtained with the eukaryotic-specific primer set.

Two oligonucleotide probes, BrB-754 and BrB-1461, targeted to variable regions of the BrB ciliate 18S rRNA sequence, were designed using the probe design algorithm of the ARB package (27) and checked against the GenBank database (3). Alignment and comparison of closely related 18S rRNA sequences demonstrated mismatches for both probes (Fig. 3). Fixed ciliate samples (15) were filtered onto 0.22-μm-pore-size white Isopore membrane filters (Millipore) by use of a gentle vacuum and washed five times with 1 ml of filtered sterile seawater. Membranes were covered with hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 8], 0.01% sodium dodecyl sulfate, 30% [vol/vol] formamide) and the appropriate fluorochrome-labeled oligonucleotide probe (50 μg). All hybridizations were conducted at 46°C for 3 h, after which membranes were floated into prewarmed wash buffer (0.102 M NaCl, 20 mM Tris-HCl [pH 8], 0.01% sodium dodecyl sulfate, 5 mM EDTA) at 48°C for 10 min to remove excess and nonbound oligonucleotide probes. Air-dried filters were mounted in an antifading gel (Biomedia, ProSciTech) before being viewed and imaged on a Bio-Rad MRC-1024 confocal laser scanning microscope (40).

Hybridizations with the eukaryote-specific probe EUK1195 (16) resulted in the presence of a fluorescence signal for both the ciliate and the internalized *Symbiodinium* sp. (Fig. 4a). Comparative hybridizations of the BrB tissue with probes BrB-754 and BrB-1461 resulted in a fluorescent signal for the ciliate, correlating the retrieved 18S rRNA gene sequence with the distinct morphological characteristics of the BrB ciliate (Fig. 4b and c). Morphological features of the ciliates could also be distinguished, including the buccal cavity on the ventral side (Fig. 4b). No signal associated with *Symbiodinium* sp. was observed for the BrB ciliate-targeted probes, supporting probe specificity. Signals from the EUK1195, BrB-754, and BrB-1461 probes were clearly distinguishable from autofluorescence signals achieved with negative-control hybridizations (NONEUB nonsense probe) (4) (Fig. 4d).

Ciliates belonging to the Scuticociliatia subclass are abundant in marine habitats and often observed as endosymbionts in marine invertebrates such as echinoids, crustaceans, polychaetes, and bivalve mollusks (25). Although the feeding behavior of the brown band ciliate requires further study, the high density of *Symbiodinium* cells observed within its membranes (Fig. 1c) is the primary cause of the brown color that characterizes the disease's appearance in the field (Fig. 1a). At present, it is unknown whether the ciliate ingests zooxanthellae in the course of feeding on live coral tissue (i.e., is carnivorous), ingests zooxanthellae in the course of feeding on dead coral tissue (histophagous), or acquires them from elsewhere (algivorous). However, the presence of high densities of feeding ciliates, in combination with the retrieval of *Symbiodinium* 18S rRNA gene sequences from DNA extracted from ciliate

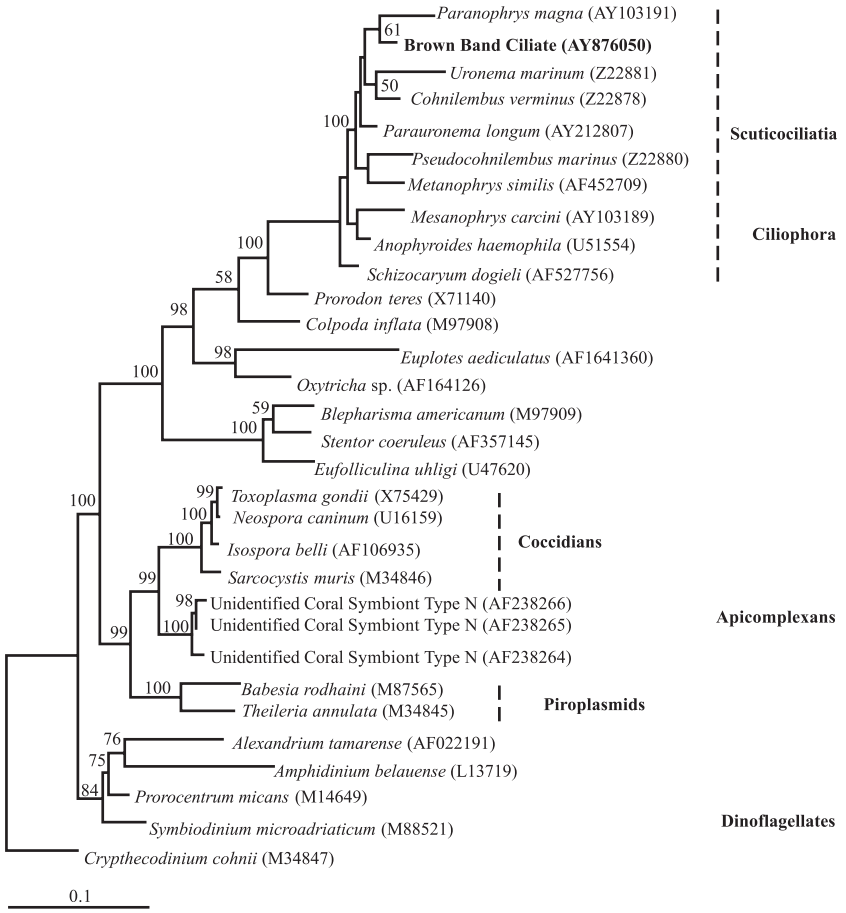

FIG. 2. Phylogenetic tree showing the relationship between the 18S rRNA gene ciliate clone sequence obtained from a sample of the coral *Acropora muricata* infected with BrB and chosen reference ciliate sequences inferred by maximum-likelihood analysis. Near-complete (>1,680 bp) 18S rRNA gene sequences were used to construct the tree. The sequenced BrB ciliate clone is denoted in boldface type. GenBank accession numbers are presented for all sequences. Branching points with >50% bootstrap support (1,000 replicates) have the values shown at the nodes. The scale bar represents approximately 10% estimated sequence divergence. The outgroup used in the construction of this tree was the dinoflagellate *Crypthecodinium cohnii*. A complete 18S rRNA gene sequence of the unidentified ciliate was obtained using conserved eukaryotic primers NS3, NS4, NS5, and NS7 (24). Sequence alignments and phylogenetic comparisons were performed using the ARB software package (26). The confidence of branch points was tested by bootstrap analyses (1,000 replicates) using the SEQBOOT and CONSENSE programs from the PHYLIP version 3.6 program package (12; PHYLIP version 3.6, 2005 edition [distributed by the author, J. Felsenstein, Department of Genome Sciences, University of Washington, Seattle]) using filtered sequences exported from the ARB package.

samples that match the clade C lineage, previously shown to be the dominant type associated with *Acropora muricata* at our sampling locations (39), suggests that members of the *Symbiodinium* are derived from coral tissue. Hence, the ciliates are likely to be either carnivorous or histophagous. Recent studies demonstrated that the internalized *Symbiodinium* zooxanthellae remain photosythetically competent within the ciliate, allowing the protozoan to gain additional energy from photosynthates and alleviate potential oxygen limitations due to high population densities and respiration demands in the brown

TABLE 1. PCR primers and 18S rRNA-targeted oligonucleotide probes used in this study

Primer or probe[a]	Sequence (5′–3′)	Target organism(s)	% Formamide	Reference or source
18S-6-CIL-V	AAYCTGGTTGATCCTGCCAG	Eukaryotes	N/A[b]	15
18S-1511-CIL-R	GATCCWTCTGCAGGTTCACCTAC	Eukaryotes	N/A	15
BrB-F-171	TCAAACCCGACTTTACGGAAG	BrB ciliate	N/A	This study
BrB-R-1721	TGCAGGTTCACCTACGGAAAC	BrB ciliate	N/A	This study
EUK1195	GGGCATCACAGACCTG	Eukaryotes	30	16
BrB-754	GTATTCGAGCCAAAGCCT	BrB ciliate	30	This study
BrB-1461	CGTATCCTTCCGGAACAGGT	BrB ciliate	30	This study
NONEUB	ACTCCTACGGGAGGCAGC	Noncomplementary	30	4

[a] Probes labeled with the indocarbocyanine fluorochrome Cy5 (Thermo Hybaid, Germany).
[b] N/A, not applicable for PCR primers.

(A) *BrB-754*

Probe Sequence	**3' - T C C G A A A C C G A G C T T A T G - 5'**
Target Sequence	**5' - A G G C T T T G G C T C G A A T A C - 3'**

Paranophrys magna (AY103191)	· · · · · a · · · · · · · · · · · · ·
Uronema marinum (Z22881)	· · · · · a a · · · · · · · · · · · ·
Cohnilembus verminus (Z22878)	· · · · · a a · · · · · · · · · · · ·
Parauronema longum (AY212807)	· · · · · a a · · · · · · · · · · · ·
Pseudocohnilembus marinus (Z22880)	· · · · · a a · t · · · t · · · · · ·
Metanophrys similis (AF452709)	· · · · · a a · · · · · · · · · · · ·
Mesanophrys carcini (AY103189)	· · · · · a a · · · · · · · · · · · ·
Anophyroides haemophila (U51554)	· · · · · a a · · · · · · · · · · · ·
Shizocaryum dogieli (AF527756)	· · · · · a a · · · · · · · · · · · ·
Prorodon teres (X71140)	· · · · · g · · t · · a t · · · · · ·
Colpoda inflata (M97908)	· · · · · a a · n · · · t · g · · · ·
Euplotes aediculatus (AF1641360)	· · · · · g · g c · · g · · · · · · ·
Symbiodinium microadriaticum (M88521)	· a · · · g · g c · · · t · · · · · ·

(B) *BrB-1461*

Probe Sequence	**3' - T G G A C A A G G C C T T C C T A T G C - 5'**
Target Sequence	**5' - A C C T G T T C C G G A A G G A T A C G - 3'**

Paranophrys magna (AY103191)	t · · · · · c · t · · · · a · g · · t ·
Uronema marinum (Z22881)	· · · · · g · t · · · · · · a · · t · ·
Cohnilembus verminus (Z22878)	· · · · · g · t · · · · · · a · · t · ·
Parauronema longum (AY212807)	g · · · · · g c · · · · · · · · g · t · ·
Pseudocohnilembus marinus (Z22880)	t · · · · · a · · · · · · · · · · t · ·
Metanophrys similis (AF452709)	t · · · · · g c · · · · · · · · g · t t ·
Mesanophrys carcini (AY103189)	t · · · · · g c · t · · · · a · g · t · ·
Anophyroides haemophila (U51554)	t · · · · · g c · · · · · · · · g · t · ·
Shizocaryum dogieli (AF527756)	· · · · · · c · · · · · · · · g a · · ·
Prorodon teres (X71140)	c · · · · · c · · · · · · · · g · · t ·
Colpoda inflata (M97908)	t · · · · · a c t · · · · · · · g · t · ·
Euplotes aediculatus (AF1641360)	· · · · · c · · · · a · · t a g a c g ·
Symbiodinium microadriaticum (M88521)	· · · · · g c · t · a · · t · g c t g ·

FIG. 3. Different sequence alignments of probes BrB-754 (A) and BrB-1461 (B). Nucleotides are only identified for mispairings; pairings are indicated by dots. The bases of the mismatches refer to the sequences of the target organism and not of the probe. GenBank accession numbers of each organism are presented in parentheses.

band zone (38). Such a mixotrophic strategy is common among freshwater oligotrichs, with enslaved photosynthetic components remaining functional for hours to days, thereby providing nutrients, covering respiratory demands, and increasing growth efficiency (11, 34–36). Whether a similar relationship exists between BrB ciliates and internalized *Symbiodinium* zooxanthellae has yet to be determined; however, symbiotic relationships between ciliates and zooxanthellae have previously been reported for ciliates living in association with corals (26).

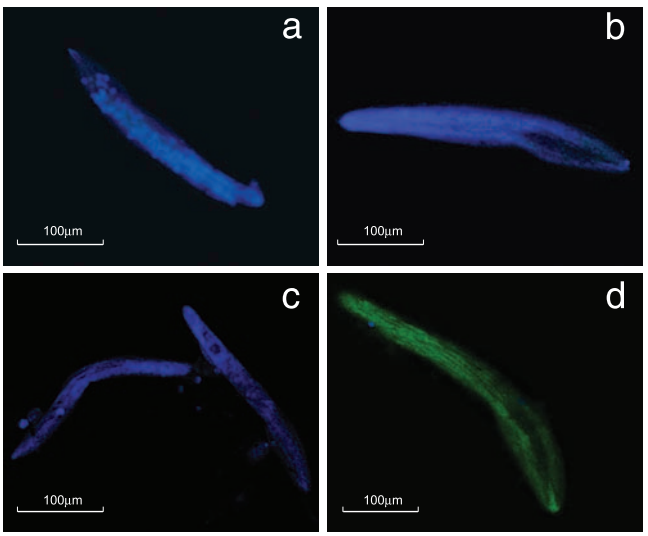

FIG. 4. Fluorescence in situ hybridization micrographs of BrB ciliates. (a) Ciliate probed with Cy5-labeled eukaryote-specific probe EUK1195 (16). (b) Ciliate probed with Cy5-labeled BrB-754-specific probe. (c) Ciliates probed with Cy5-labeled BrB-1461-specific probe. (d) Ciliate probed with Cy5-labeled NONEUB nonsense probe, demonstrating autofluorescence of only the sample-specific probe.

Although common in marine environments, ciliates are rarely classified as pathogenic parasites (28), especially in coral communities. One study has linked a GBR coral disease with the *Halofolliculina corallasia* heterotrich ciliate. Known as skeletal eroding band, this disease has been characterized by an advancing mass of ciliates whose pericytostomial wings are encased within flask-like black loricae (5). Protozoan infections have also been identified on corals held in aquaria. For example, the consumption of coral tissue by the ciliate *Helicostoma nonatum* produces brown jelly-like symptoms in infected aquarium corals (7). Willis et al. (42) speculated that the ciliate associated with BrB might be related to *H. nonatum*, although results from this study suggest that it belongs to a different family. Other studies have identified a protozoan belonging to the phylum Apicomplexa within microbial communities associated with the coral *Montastraea annularis* in the Caribbean, but although this protozoan is related to a group of highly parasitic organisms, whether or not it is parasitic on corals is currently unknown (37).

The causative agent of the coral disease BrB remains unknown. The appearance of a white bleached zone, often observed between healthy coral tissue and an advancing mass of ciliates (Fig. 1a), suggests that the ciliate may invade secondarily after coral health is compromised, although it is clear that

the ciliate subsequently becomes responsible for macroscopic field signs of BrB disease. A number of factors may compromise coral health, including bacterial or viral infections, injury, and, alternatively, apoptosis triggered by stress, injury, or infection (2, 18). As the health of the coral deteriorates, necrosing tissue could attract the ciliate to feed on both bacteria and zooxanthellae associated with dead and dying coral tissue. At high densities, however, the ciliates may become the primary cause of tissue loss as they uptake photocompetent zooxanthellae to alleviate potential oxygen limitations (38).

In summary, the characteristic macroscopic signs of the coral disease BrB have been attributed to the presence of a newly identified ciliate species of the class Oligohymenophorea, subclass Scuticociliatia. Future studies investigating the life cycle and taxonomic traits of the ciliate are required along with additional microbiological studies to further clarify the nature of the causative agent(s) of this coral disease.

Nucleotide sequence accession number. The nucleotide sequence data have been submitted to the GenBank nucleotide sequence database under accession number AY876050.

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