FEATURE ARTICLE

First description of algal mutualistic endosymbiosis in a black coral (Anthozoa: Antipatharia)

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ABSTRACT: The class Anthozoa is the largest metazoan group forming mutualistic symbioses with microalgae. These algal symbionts (most commonly dinoflagellates of the genus *Symbiodinium*) are distributed across most anthozoan orders. Records of algal cells in antipatharian (black coral) tissues have been reported, but no detailed descriptions of a mutualistic endosymbiosis exist. Here we report on zooxanthellate specimens of an unidentified black coral species within the genus *Cirrhipathes* that were collected from reef slopes at depths of 15 to 38 m in the Indonesian Archipelago. Symbionts were abundant (~10⁷ symbionts cm⁻²) and ultrastructural analysis revealed the presence of a distinct symbiosome surrounding the algae, as well as algal reproduction inside the gastrodermal layer. Molecular analysis revealed the algae to be closely related to the symbionts (*Symbiodinium* clade G) of clionid sponges. There was also evidence for additional symbionts in clade C at low abundance. Taken together, these findings (high abundance, taxonomic identity, presence of symbiosome, *in situ* reproduction, and depth distribution) strongly suggest that these algae are functioning as mutualists. This study confirms and describes the symbiosis between *Symbiodinium* and a black coral species of the genus *Cirrhipathes*, supports the pervasiveness of mutualisms among anthozoan taxa, and highlights the diversity and flexibility of these symbiotic associations in a poorly studied group.

KEY WORDS: Symbiosis · Zooxanthellae · *Cirrhipathes* · *Symbiodinium* · Black corals · Coral reefs

INTRODUCTION

Antipatharians, which are commonly known as black corals, constitute a small order (~230 species) of colonial anthozoans found in all the oceans of the world, but particularly diverse and abundant in tropical and subtropical waters (Tazzioli et al. 2007). Most of the described species are restricted to deep waters; hence, their ecology is poorly understood.

Black corals have been considered the most primitive group of hexacorallians (zoantharians) due to their simple morphological organization involving 6 tenta-
icles, and a generally reduced muscular system which allows only partial contraction of the tentacles (van Beneden 1897, Brook 1889, von Koch 1889, van Pesch 1914, Gravier 1921). In the past, these organisms were included (together with ceriantharians) in the taxon Ceriantipatharia because of their similarity with the cerianthic larval stages ‘antipathula’ and ‘cerinula’ (van Beneden 1897, Larink & Westheide 2006). However, recent molecular studies based on mitochondrial DNA support the Order Antipatharia as a distinct evolutionary group that is separate from the Ceriantharia (Brugler & France 2007).

Black corals have been considered azooxanthellate—a view likely resulting from the deep water (>100 m) location and preference for low-light conditions of most known specimens (Grigg 1965). However, records of black corals hosting algal cells are reported in the literature.

The first record of algal cells in black corals dates back to 1889, when Brook described yellowish green bodies of irregular outline within the hyaline cells of the ectoderm of Tylopathes crispa Brook, 1889. Subsequently, van Pesch (1914) listed 7 Indo-Pacific species hosting algae in their tissues, including 3 species of the genus Cirrhipathes (C. anguina [Dana, 1846], C. contorta [van Pesch, 1910] and C. spiralis var. striata [van Pesch, 1910]), 3 of the genus Stichopathes (S. gracilis [Gray, 1857], S. saccula [van Pesch, 1910], and S. variabilis [van Pesch, 1914]), and one species of dubious systematic position (Cirrhipathes [Hillopathes] ramosa [van Pesch, 1910]).

These historical reports are in partial agreement with what is currently known regarding algal symbiosis in other anthozoan groups. Algae were reported in the gastroderm of tentacles, the body wall and actinopharynx (in Cirrhipathes anguina, C. spiralis var. striata, Stichopathes gracilis, S. variabilis and S. saccula), in the ectoderm of the tentacles (S. variabilis) or in the mesenteries (C. contorta and S. saccula). The algal cells, which were spherical or pear-shaped and brown or yellow-greenish in color, were generally 5 to 10 µm in diameter (Brook 1889, van Pesch 1914). However, some inconsistencies concerning the localization of cells, their abundance and size, and the collection depth of the specimens did not unequivocally establish the algal cells as symbionts. For example, algal cells were found only in portions of the gastroderm, and their abundance was always low (except in C. ramosa, where numerous colorless cells were reported to be crowded together in the gastroderm of polyps). Cells were also sometimes unusually small (algal cells in the tentacles, oral cone and body wall ectoderm of C. anguina and S. gracilis were only 3 µm in diameter, compared to a standard size range of ~8 to 12 µm in Symbiodinium). Finally, specimens of 2 of the species were found in unusually deep waters (~560 m for S. saccula and 730 m for Tylopathes crispa). By modern standards, however, the information provided by Brook (1889) and van Pesch (1914) is not definitive, both in terms of the systematic description of the partners involved, and the evidence for a symbiotic relationship between them.

The only contemporary record of zooxanthellate black corals is that of Wagner et al. (2010), who reported algal cells in 10 species of Hawaiian antipatharians collected at depths of 10 to 396 m. Dinoflagellate algae of the genus Symbiodinium were found at relatively low densities (0 to 92 cells mm⁻³) in the gastrodermal tissues of these corals. The great depths at which some of these specimens were collected exceeded those previously reported for zooxanthellate corals; e.g. the scleractinian Leptoseris hawaiiensis Vaughan, 1907 was found at 165 m at Johnston Atoll in the Pacific (Maragos & Jokiel 1986), and a scleractinian coral collected by dredge was found at a depth of 182 to 212 m off Key Largo in Florida (Zahl & McLaughlin 1959).

During a survey of the black coral communities at 2 different sites in the Indonesian Archipelago, we collected specimens of an unidentified species of Cirrhipathes hosting a large number of zooxanthellae in the gastroderm of its polyps. The purpose of this study is to report and describe a case of algal endosymbiosis in black corals using contemporary methods, namely light microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and molecular techniques.

**MATERIALS AND METHODS**

**Sample collection and preparation.** Two specimens (Ment15 and Ment52) were collected by scuba divers in July 2007 at a depth of 38 m on the reef slopes around Siburu Island in the Mentawai Archipelago, Indonesia (1° 58’ 31.67” S, 99° 36’ 8.36” E). A third specimen (Indo 25) was collected in July 2008 at 15 m depth on the vertical wall of Siladen in the Bunaken Marine Park (1° 37’ 37.90” N, 124° 48’ 6.32” E). Colonies were photographed in situ prior to collection, and samples of each specimen were preserved in 95% ethanol for molecular analysis, as well as 4% formaldehyde for morphological analysis. For ultrastructural investigation, parts of each sample were fixed for 12 h in 2.5% glutaraldehyde buffered with filtered sea water (pH adjusted to 7.5 to 7.8 with 0.1 N NaOH), then repeatedly rinsed in the same buffer and stored at 4°C.

The morphological analysis of the black coral samples was carried out with a stereo microscope (Zeiss...
**Symbionts:** The ITS-2 region of algal rDNA was amplified using the forward and reverse primers of LaJeunesse (2001), with the addition of a GC-clamp on the reverse primer for use with DGGE. PCR parameters were the same as the antipatharian parameters (see 'Molecular analyses. Host' above), except for an annealing temperature of 55°C. DGGE gels were run using a 35 to 75% gradient. Visible bands were excised, re-amplified with the same primers (reverse primer without clamp), sequenced, and edited as described in 'Molecular analyses. Host' above. Genotypes for each sequenced band were obtained by Basic Local Alignment Search Tool (BLAST) searches in GenBank (www.ncbi.nlm.nih.gov), and named according to the *Symbiodinium* nomenclature of LaJeunesse (2001).

**Molecular cloning.** In order to confirm the identity of the ITS-2 *Symbiodinium* genotypes found using DGGE, and to determine their phylogenetic placement, the 5'-end of the large subunit (LSU) rDNA was amplified using primers from Baker et al. (1997) for the D1–D2 regions. Thermocycler parameters were as described in 'Molecular analyses. Host' above. Genotypes for each sequenced band were as described. Amplified products were sequenced, and edited as described in 'Molecular analyses. Host'.

**Phylogenetic reconstruction.** Phylogenetic analyses for the antipatharian ITS-1, 5.8S, and ITS-2 alignment were performed using Bayesian maximum likelihood (MB) in MrBayes v3.1.2 (Huelsenbeck & Ronquist).
for *Symbiodinium* simplex (Lohmann 1908) (EF205014) was used to examine the presence of numerous zooxanthellae cells in the gastroderm. (J, K) SEM images of the algal cells within the cnidarian gastrodermic tissue. Scale bars: A = 10 cm; B,C = 2 mm; D,F,H = 1 mm; E,G = 100 µm; J,K = 20 µm; I = 10 µm

**RESULTS**

**Morphology of black coral specimens**

Based on traditional morphological characters, all 3 specimens belong to the same unidentified species in the genus *Cirrhipathes*, and are characterized by unbranched, unpinnulated colonies, and by polyps that are irregularly arranged around the stem. The colonies of the symbiotic specimens have a 0.5 to 1.5 m long straight corallum. In the longest specimens, the corallum is slightly coiled at the apex (Fig. 1A). The polyps are large (1 to 1.5 mm in transverse diameter, interpolypar distance 0.5 to 1.1 mm) with a prominent oral cone (0.3 mm high). Sagittal tentacles are slightly longer than the laterals, and are 1 to 4 mm long, but their length varies greatly depending on contraction state (minimum 0.6 to 0.7 mm long). A white transparent ectoderm over a gastrodermal layer of yellow-brown zooxanthellae contributes to an overall velvety appearance (Fig. 1B,D). The long tentacles are characterized by circular spots of cnidocysts that are uniformly distributed over their surface (Fig. 1D,E). The cnidome is similar among the 3 analyzed specimens: spirocysts (15 × 2 µm), basitrich isorhizae (20 to 25 × 3 µm) and mastigophores (15 × 5 µm). The spines (250 to 300 µm high, 120 µm wide and 350 to 400 µm apart) are arranged in 6 to 8 longitudinal rows, are equal in shape along the entire length of the stem and on both sides of it, and are triangular, with a smooth or slightly papillose surface (Fig. 1F).

Sections examined using fluorescence microscopy provided evidence for the localization of the zooxanthellae inside the gastroderm of the tentacles (Fig. 1G), and in the gastroderm of the gastric cavity (Fig. 1H). Fractured polyps observed under SEM showed that algal cells were evenly distributed in rows even down the length of the stem (Fig. 1I–K).

**Morphology of zooxanthellae**

Histological sections of the polyps confirmed the presence of numerous zooxanthellae in the gastroderm.
of the tentacles (Fig. 2A, and inset) and in the gastroderm of the mesenteries of the oral cone (Fig. 2B). In both cases, these algal cells were evenly arranged inside the tissue, where they were easily distinguishable by their uniform, spherical, and compact shape. In sections for ultrastructural analysis, zooxanthellae were clearly visible inside discrete cell vacuoles and were distributed in rows in the gastrodermal tissue (Fig. 2C). All vacuoles were delimited by a symbiosome membrane (Fig. 2F) (Wakefield et al. 2001), which separated the symbionts from the cytoplasm of the host cells. Algal cells measured 5 to 7 µm in dia-
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meter, and were present at a density of \( \sim 10^7 \) cells \( \text{cm}^{-2} \), including those distributed in the interpolylar coenenchyme. They showed the typical characteristics of symbiotic dinoflagellates: peripheral thylakoids formed by a series of superimposed dark lamellae (Fig. 2F, inset); a large rounded accumulation body or lipid body (Fig. 2D); and a pyrenoid body surrounded by a layer of starch grains (Fig. 2D, inset). Some images revealed the occurrence of 2 algal cells inside the same vacuole—a feature consistent with a mitotic division phase (Fig. 2E).

**Phylogenetic reconstruction of studied antipatharians**

Phylogenetic reconstruction resulted in a tree topology similar to that shown by Lapian et al. (2007) and Bo et al. (2009) (Fig. 3). The 2 zooxanthellate antipatharians (Ment15 and Ment53) grouped monophyletically, and fell within a large clade (well supported by MP, less well supported by MB, ML and ME) that contained 6 of the 7 members analyzed from the family Antipathidae, and 1 of the 3 members of the Aphanipathidae, *Rhipidipathes reticulata* (Esper, 1795). Ment15 and Ment53 grouped as a sister clade to *R. reticulata* in all reconstruction methodologies, except MB, with reasonable statistical support (ML 72, MP 80, ME 92); and the Ment15-Ment53-*R. reticulata* clade was a sister clade to a large Antipathidae clade (excluding *Pseudocirrhipathes mapia* Bo & Bavestrello, 2009).

All analyzed members of the family Myriopathidae formed a monophyletic clade that is well supported by all reconstruction methodologies. The remaining 2 species belonging to the family Aphanipathidae, *Phanopathes rigida* (Pourtalès, 1880) and *Aphanipathes cf. sarothamnoides* Brook, 1889, grouped together. However, the family remained polyphyletic with respect to the other member of the family, *Rhipidipathes reticulata*. Specimens of *Pseudocirrhipathes mapia* did not group with the other members of the family Antipathidae, but formed a weakly supported sister clade to a large clade that contains the Myriopathidae clade, and *P. rigida* together with *A. cf. sarothamnoides* as subclades.

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**Fig. 3.** Cladogram showing supported clades for Bayesian maximum likelihood, maximum likelihood, maximum parsimony and minimum evolution methods that have at least one reconstruction method supported with a bootstrap value or posterior probability >50 (values shown on branch nodes, respectively). Branch lengths do not represent distances. **Bold:** zooxanthellate black coral samples. Vertical lines: taxa grouped at the family level. Dotted line: uncertain systematic placement of samples Ment15 and Ment53.
Molecular and phylogenetic analysis of *Symbiodinium*

DGGE analysis of both zooxanthellate black coral samples (Ment15 and Ment53) produced a band profile with one dominant band flanked by fainter secondary bands (Fig. 4). Sequence analysis in BLAST revealed the dominant band to be 95% (286/300 bp) similar to G3 (accession no. AM748600). Of the secondary bands, one was found to be 99% (1 bp difference) similar to C15 (C→T at position 31) and C4 (insertion of T at position 245). The amount of clade G relative to clade C appeared to be greater based on the former’s darker band intensity, although this is only weak evidence for greater abundance of symbiont clade G relative to clade C. The other faint secondary bands did not produce clean sequences usable for contig assembly.

Cloning of LSU rDNA produced sequences that closely matched those of members of clade G *Symbiodinium*. RFLP analysis revealed a single profile with both TaqI and HhaI (Fig. 5) that matched the profile predicted from the clade G LSU sequences. Phylogenetic reconstruction (Fig. 6) of the LSU rDNA sequences placed all sequences from both black coral samples, as well as the clionid sponge symbiont sequences, in a well-supported clade, using both MB and ML (although bootstrap support was low with ML). The remaining tree topology was similar to that of Pochon et al. (2004, 2006), Schönberg & Loh (2005), and Pochon & Gates (2010).

DISCUSSION

In cnidarians, symbiotic relationships with zooxanthellae of the genus *Symbiodinium* have been recorded in the classes Hydrozoa, Scyphozoa, and Anthozoa (Baker 2003). Among anthozoans in particular, the phenomenon is exceptionally widespread, both in octocorals (alcyonarians) and hexacorals. The subclass Hexacorallia (Zoantharia) includes many zooxanthellate cnidarians in the Scleractinia, Actinaria, Corallimorpharia, and Zoanthidea (Stat et al. 2006). Until recently (Wagner et al. 2010), however, antipatharians (like ceriantharians) were traditionally considered to be azooxanthellate hexacorallians, probably as a result of various factors such as the distribution of many of these organisms in deep water. Wagner et al. (2010) reported low densities of *Symbiodinium* in several Hawaiian deep-water antipatharians, in agreement with historical reports of sparse algal cells in some black corals (Brook 1889, van Pesch 1914). Low and

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**Fig. 4.** DGGE analysis of internal transcribed spacer ITS-2 rDNA showing characteristic profile for *Symbiodinium* found in black coral samples Ment15 and Ment53. The dominant band (2) was a 95% match to G3. The secondary band (1) was a 99% match to C15 and C4 (see text for details). All other secondary bands did not sequence cleanly for accurate contig assemblies.

**Fig. 5.** Restriction fragment length polymorphisms (RFLPs) in *Symbiodinium* large subunit rDNA, using the enzymes TaqI (left image) and HhaI (right image). Each sample was independently amplified and digested twice. Lane 1: Ment15, Lane 2: Ment53, Lane 3: Ment15, Lane 4: Ment53. Left lane for each image is 100 bp size standard. Only one RFLP genotype (corresponding to a *Symbiodinium* in clade G) is detectable in each sample.
Fig. 6. Bayesian phylogram showing placement of algal symbionts in black coral samples Ment15 and Ment53 (in **bold**) among the representative *Symbiodinium* clades A–I using partial large subunit rDNA sequences. Bayesian posterior probabilities and maximum likelihood (ML) bootstrap replicates (1000 reps) are shown on branch nodes, respectively. Values <50 are shown as ‘–’. Taxa include the name of *Symbiodinium* clade, and their host and GenBank accession number in parentheses.
variable cell densities suggest that some species may follow a potentially mixotrophic lifestyle involving facultative algal symbiosis that may be dependent on location and environment. These factors have probably led to an underestimation of algal symbiosis in antipatharians in general.

If we consider all literature records of zooxanthellate black coral species, then algal symbionts are evenly distributed in both whip (10 species: 5 Cirrhipathes and 5 Stichopathes) and branched (7 species: 2 Antipathes, 1 Aphanipathes, 2 Myriopathes, 1 Acanthopathes, 1 Bathypathes) antipatharians (4 out of 7 antipatharian families). However, this is the first report that provides multiple lines of evidence supporting a genuinely mutualistic symbiotic relationship, and it involves just one species in the genus Cirrhipathes. The observations of Brook (1889) and van Pesch (1914), although historically significant, have not been corroborated by sufficient evidence to support, by contemporary standards, the existence of a symbiotic relationship. Similarly, Wagner et al. (2010), while greatly expanding our appreciation for algal diversity in a variety of antipatharians, indicated that the available evidence (low cell densities, extreme depths) suggested that the algae might be involved in parasitic rather than mutualistic symbiosis. Our specimens contained very high densities of zooxanthellae inside the gastroderm of both tentacles and gastric cavities, and their organizational pattern, as well as the presence of mitotic algal cells inside the same vacuole, suggests that these cells are capable of reproducing inside their host. The occurrence of a membrane layer delimiting the zooxanthellae is also interpreted as indicating an endosymbiotic association with these black corals. These membranes are typical of cnidarian–dinoflagellate symbiosis, and the use of monoclonal antibodies has shown that these membranes are symbiont-derived and are not present when symbionts are maintained in culture (Wakefield et al. 2001).

We found evidence for symbionts in 2 clades of Symbiodinium in the black coral samples. However, the DGGE bands from which C15-like and C4-like ITS-2 sequences were recovered were faint, no RFLP profiles corresponding to clade C were observed, and no C-sequences were recovered from cloning of LSU rDNA. Consequently, we conclude that the dominant symbionts of this antipatharian are very similar to the symbionts in clade G found in clionid sponges (Schönberg & Loh 2005, Granados et al. 2008). It is possible that the C-sequences we retrieved using DGGE represent symbionts which are preferentially amplified by ITS-2 primers and are thus detectable by DGGE, but which are too rare to be picked up by the cloning methodology used here for LSU rDNA. These findings are in contrast to Wagner et al. (2010), who found that Hawaiian black corals hosted members of clade C Symbiodinium, including some types found in Hawaiian zooxanthellate scleractinian corals. Our Indonesian Cirrhipathes sp. hosts members of clade G Symbiodinium that are very different from those found in scleractinian corals worldwide (Baker 2003, but see LaJeunesse et al. 2010 for a report of some scleractinians in intertidal environments in Thailand hosting members of clade G).

The unusual symbionts that dominate these black corals, and which are also found in some sponges, are most closely related to (but distinct from) the Symbiodinium in clade G that have been found in some foraminiferans (genus Marginopora) (Pochon et al. 2001). We have differentiated these 2 groups within clade G by referring to the symbionts initially described from Foraminifera as members of subclade G1, and the symbionts of black corals and clionid sponges as members of a distinct subclade G2 (Fig. 6). This follows the precedent of Pochon et al. (2001) and Pochon et al. (2006), who introduced numerical subclades for Symbiodinium clades F and D, respectively.

The purpose of the present study is to report and describe, using contemporary methods, a case of algal endosymbiosis in black corals. In some cases, the presence of zooxanthellae has been used as a taxonomic character for the description of new cnidarian species (e.g. Marques et al. 2000). However, we have chosen not to formally describe a new species of Cirrhipathes here due to the complex and unresolved taxonomy of the whip black coral taxa. Moreover, although the morphological characters of our specimens clearly support their inclusion in the genus Cirrhipathes, the molecular analyses indicate that they do not belong to the Cirrhipathes clade of Lapian et al. (2007) and Bo et al. (2009) (Fig. 3).

In conclusion, these findings (high abundance, taxonomic identity, presence of symbiosome, in situ reproduction, and depth distribution) strongly suggest that these algae are functioning as mutualists. The present study documents the symbiosis between Symbiodinium of the subclade G2 and a black coral species of the genus Cirrhipathes, supports the pervasiveness of mutualisms among anthozoan taxa, and highlights the diversity and flexibility of these symbiotic associations in a poorly studied group.

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