Deep sea treasures - insights from museum archives shed light on coral microbial diversity and functioning within deepest ocean ecosystems

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Abstract

Deep sea benthic habitats are low productivity ecosystems that host an abundance of organisms within the Cnidaria phyla. The technical limitations and the high cost of deep sea surveys have made exploring deep sea environments and the biology of the organisms that inhabit them challenging. In spite of the widespread recognition of Cnidaria's environmental importance in these ecosystems, the microbial assemblage and its role in coral functioning have only been studied for a few deep water corals. Here we explored the prokaryotic diversity of deep sea corals by recovering nucleic acids from museum archive specimens. Firstly, we amplified and sequenced the V1-V3 regions of the 16S rRNA gene of specimens held in museum archival storage and utilised these to shed light on the microbial diversity associated with seven families of corals collected from depth in the Coral Sea (depth range 1,309 to 2,959 metres) and Southern Ocean (depth range 1,401 to 2,071 metres) benthic habitats. Most surprisingly, Cyanobacteria were consistently associated with six out of seven coral families from both sampling locations, suggesting that these bacteria are potentially ubiquitous members of the microbiome within these cold water and deep sea organisms. Consistent with previous studies, we show that the bacterial phyla Proteobacteria, Verrucomicrobia, Planctomycetes and Acidobacteriota dominate the microbial community of corals in the deep sea. We also explored the genomes of the genus *Mycoplasma*, which we identified as associated with specimens of three deep sea coral families, finding evidence that these bacteria may aid the host immune system. Importantly our results show that museum specimens retain components of host microbiome that can provide new insights into the diversity of deep sea coral microbiomes (and potentially other organisms), as well as the diversity of microbes writ large in deep sea ecosystems.

Introduction

The deep seafloor ecosystem (> 1,000 m of depth) is one of the most remote, unproductive and largest ecosystems on the planet [1], it is mainly unexplored, covering approximately 60% of Earth’s solid surface [2]. The impact of anthropogenic activities on the biota of the deep sea has been described as catastrophic [3]. It has been estimated that between 1997 and 1999 around 50 tons per year of sessile fauna were removed from the seafloor of Alaskan waters by trawling activities [3]. Similarly, 95% of the large sessile fauna in fished seamounts south of Tasmania has been reported as impacted by the activities of bottom trawlers [4]. Deep sea ecosystems are considered degraded environments and data available to date suggest that these systems are losing their biodiversity [2]. As such researchers suggest that deep sea sessile organisms are increasingly endangered and given the challenges in studying these largely inaccessible habitats, any retrievable information on their biology, function, and contribution to ecosystem stability is highly valuable.

The remoteness of the deep sea ecosystem hinders our understanding of many aspects of deep sea organisms' biology. Among them are deep sea cold water corals, which encompass stony corals (Scleractinia), soft corals (Octocorallia), black corals (Antipatharia), and hydrocorals (Stylasteridae) [5]. These organisms have been reported across the oceans worldwide [3, 5, 6] and their biodiversity in the
deep ocean is greater than that of shallow water ecosystems [3, 6]. Corals in these ecosystems occur both as isolated colonies and larger three-dimensional reef structures that provide habitat, refuge and nursery grounds to a wide variety of organisms including commercially important species [5]. Importantly, growth rates of deep water coral species are lower than their shallow water counterparts [7, 8] making deep sea reef structures particularly vulnerable to direct anthropogenic disturbances.

Unlike most reef forming shallow water corals, cold and deep water corals lack symbiotic dinoflagellates that provide photosynthetic byproducts that support coral growth [9]. Accordingly, we hypothesize that the microbial associates of deeper water corals may play crucial roles in the nutrient acquisition, such as recycling nutrients or degrading recalcitrant organic matter [10–12]. For instance, the deep sea scleractinian coral *Desmophyllum pertusum* (previously known as *Lophelia pertusa*) relies on symbiotic bacteria to obtain fixed nitrogen [12] and the same process has been postulated for octocorals, where the association with bacteria such as *Spirochaeta*, *Bacillus* and *Propionibacterium* might facilitate the nitrogen metabolism of the holobiont [8, 13]. In fact, several molecular surveys suggest that cold and deep water corals maintain species-specific prokaryotic communities [14–16]. Indeed, anatomical compartments such as the coral mucus, tissue and when present skeleton host specific prokaryotic communities [14–16], as has been observed in their shallow water relatives [17–19]. Currently, the number of known cold and deep water coral species exceeds 2,500 [6], but despite their prokaryotic associates' recognised importance, only the microbiomes of a few taxa have been characterised.

Museums worldwide hold specimens and collections derived from decades of deep sea exploration. Many of these collections include cold and deep water coral specimens. These specimens naturally include an abundance of microorganisms and symbionts whose biology remains unstudied. A better understanding of the role of these organisms could yield a plethora of insights into these associations and ecosystems. Given the rapid loss of biodiversity in the Anthropocene as well as the technical and logistical difficulties in studying the deep sea, specimens held in collections could provide new insight into these ecosystems, their assemblages and function. Here we investigated the microbial diversity of cold and deep water corals found in museum collections, as a means to generate hypotheses on the functional roles of the prokaryotic partners, to expand our understanding of cold and deep water coral microbiomes, and to assess the utility of using museum specimens as a tool to investigate deep water organismal and ecosystem function.

**Material and Methods**

**Sample collection and processing**

The museum archival specimens analysed in the current study were taken from two collections undertaken in deep sea ecosystems of Australia. Specimen information are available at Supp. Table 1. Firstly, samples were collected at seamount locations in the Tasman fracture southwest of Tasmania at depths ranging from 1,309 to 2,959 metres between March 2007 and January 2009 by the Commonwealth Scientific and Industrial Research Organisation (CSIRO; Fig. 1). Secondly, another
collection was generated from four locations across the northeast of Australia at depths ranging from 1,401 to 2,071 metres between August and November 2020 during oceanographic explorations conducted by the Schmidt Ocean Institute R/V Falkor “Northern Depths of the Great Barrier Reef expeditions” (expeditions FK200802 and FK200930; Fig. 1). The coral collected collated by CSIRO from the Tasman Fracture included 28 specimens that had been collected from the deep sea using tucker trawls. Following collection, the corals were rinsed in filter-sterilized seawater and stored at ambient temperature in 100% ethanol. The corals collected by Schmidt Ocean Institute from the Great Barrier Reef deep sea expedition included 20 specimens collected by the ROV SuBastian using a sterilized carousel sampler, which were subsequently stored in 95% ethanol (9) or snap-frozen by immersion in liquid nitrogen (11). The taxonomy of the coral specimens was inferred from intact specimens and assigned according to their morphology and structures by coral taxonomists. However, it is important to note that the taxonomy for these species is largely unresolved at lower taxonomical levels (e.g. genus and species) in several lineages, as such specimens were identified and annotated to the family level. Also note that samples are cross referenced to museum type specimens for further taxonomic analysis as required. The specimens in our study were classified as belonging to the families Anthomastinae (1), Keratoisidinae (20), Kophobelemnidae (1), Paragorgiidae (3), Primnoidae (6), Prooptiliidae (4) and Umbellulidae (4) in the order Scleractinacea, the family Nephtheidae (3) in the order Malacalcyonacea, and the family Caryophylliidae (6) in the order Scleractinia. A fragment of the tissue of each specimen was collected inside a safety cabinet with sterile razor blades and tweezers, rinsed with DNA-free water, snap-frozen by immersion in liquid nitrogen and stored at -80°C until processing.

Library preparation, sequencing, initial quality control and subsequent analyses

The total DNA was extracted from the tissue of each specimen and four blank samples using the QIAmp® DNA Mini Extraction kit (QIAGEN). Blank samples served as control. DNA concentration and quality were initially determined using the NanoDrop 2000c, then repeated using the Qubit Fluorometer and Qubit dsDNA broad-spectrum assay kits. The V1-V3 region of the 16S rRNA gene was PCR amplified using HotStarTaq plus master mix kit (Qiagen, USA) and the primer pairs 27F [5’-AGAGTTTGATCCTGGCTCAG-3’] and 519R [5’-GTNTTACNGCGGCKGCTG-3’] with a thermal cycling profile of (i) 3 min at 94°C; (ii) 28 cycles, with each cycle consisting of 30 sec at 94°C, 40 s at 53°C, and 1 min at 72°C; (iii) a final elongation step of 5 min at 72°C. After the PCR, products were checked in 2% agarose gels and samples were pooled in equal proportions. Pooled samples were purified using Ampure XP beads and two DNA libraries were prepared following the Illumina TruSeq DNA library protocol. DNA sequencing was performed by MrDNA (Molecular Research LP; Shallowater, TX, USA) using 2 x 300-bp paired ends on an Illumina MiSeq platform following the manufacturer's guidelines and resulted in two DNA libraries.

Sequences were processed using the QIIME2 pipeline version 2020.11 [20]. Cutadapt was used to remove primers [21]. DADA2 was used to merge forward and reverse reads, remove poor-quality sequences,
perform dereplication and eliminate chimeras [22]. DADA2 on the first DNA library was used with the parameters –p-trunc-len-f 260, –p-trunc-len-r 200, –p-trim-left-f 25, –p-trim-left-r 20, –p-trunc-q 10 and –p-chimera-method “pooled”; and on the DNA library with parameters–p-trunc-len-f 260, –p-trunc-len-r 200, –p-trim-left-f 25, –p-trim-left-r 20, –p-trunc-q 0 and –p-chimera-method “pooled”. The resulting DADA2 frequency tables and taxonomy were merged with the QIIME2 in-but commands *qiime feature-table merge* and *qiime feature-table merge-taxa*, respectively. Taxonomy was assigned using the feature-classifier plugin in-built in QIIME2 SILVA v138 QIIME release [23]. Downstream analyses were conducted using R-Studio version 1.4.1717 and packages *decontam* [24], *dplyr* [25], *ggplot2* [26], *microbiome* [27], *phyloseq* [28] and *tmap* [29].

An alignment of the 16S rRNA gene of prokaryotic sequences was performed using MUSCLE [30] in Geneious Prime 2019.1.3 (https://www.geneious.com). The prokaryotic 16S rRNA gene alignment included a total of 1,298 ASVs and we also included 199 archaeal reference sequences retrieved from the latest release of the Genome Taxonomy Database (GTDB; Supp. Table 2). The alignment was cut at the minimum length of the used 16S rRNA gene amplicon (368 bp). To build the phylogenetic tree we selected the model TVMe + R10 by using the built-in model selection function in IQTree v2.2.0 [31]. The branch support was estimated by using 1,000 bootstrap replicates on the nucleotide sequences. Afterwards, the phylogenetic tree was edited in Adobe Illustrator 26.0.3 and Interactive Tree of Life [32].

We also searched *Mycoplasma* assembled genomes on the National Center for Biotechnology Information (NCBI) and downloaded 600 feature tables containing annotated coding DNA sequences. Then, we used InterPro [https://www.ebi.ac.uk/interpro; 33] to search for genes that could provide possible explanations for the association between *Mycoplasma* and CORAL.

**Results and Discussion**

**Sequencing statistics and phylogenetic inference**

After denoising ASVs filtering and contaminant removal, the 16S rRNA gene dataset consisted of 152,262 sequences of an average length of 408bp aggregated in 1,916 ASVs. According to the SILVA v138 QIIME release 1,235 ASVs were classified as Bacteria, 40 as Archaea and 641 were unassigned. To assess the reliability of our dataset and to clear the hypothesis that the detected microbial taxa were the result of contamination or sequencing biases, we performed further manual checks. For instance, we aligned the sequences of our dataset along with 199 archaeal partial (V1-V3 regions) 16S rRNA genes and removed 282 assigned and 352 unassigned sequences because they did not present a significant alignment distance with other sequences, or because they were chimeras. According to our phylogenetic analysis, none of the sequences assigned to the Archaea belonged to this domain but to the bacterial orders Sphingomonadales, Reyranellales, Rhizobiales, Rhodobacterales and Parvibaculales in the phylum Proteobacteria and to the order Ktedonobacterales in the phylum Chloroflexi. Misclassification of these sequences could have been determined by the high similarity (min: 70.3%; median: 85.9%; max: 100.0%; Supp. Table 3) of the 16S rRNA gene V1-V3 regions between these bacterial orders and the Archaea.
Thus, we performed this analysis using a subset of our dataset including 41,939 sequences shared across 1,282 bacterial ASVs that were curated according to the phylogenetic inference of our sequences.

The residual $\alpha$-diversity

By measuring the $\alpha$-diversity indices observed and Shannon, we compared the residual bacterial diversity of the coral families except for the Anthomastinae and Kophobelemnidae which were represented by only one sample each (Fig. 2a). These measurements revealed variability in the $\alpha$-diversity of the bacterial communities retrieved across specimens of the same coral family, with the Primnoidae and Umbellulidae being the least variable and the Keratosinididae and Protoptilidae showing the greatest variability across both $\alpha$-diversity indices (Fig. 2a). The $\alpha$-diversity variability measured within the families Keratosinididae and Protoptilidae can be explained by several important various factors which should be highlighted in considering the data generated and any comparisons of the coral families within the study including: 1) there is ~3,277 km between the sampling locations for the 2 expeditions that were undertaken to compile these museum collections (Fig. 1); 2) the different preservation methods of specimens (100% ethanol vs frozen); the uncertainty in taxonomic classification below family level for the specimens; and also the prolonged preservation time for some specimens in 100% ethanol at ambient temperature [34]. Acknowledging these considerations, according to the Shannon index, which takes into account both richness and evenness [35, 36], the family Umbellulidae had, on the median, the most diverse and evenly distributed prokaryotic communities (Fig. 2a), whilst the Primnoidae, on the median, had the least diverse bacterial community dominated by few taxa (Fig. 2a). However our data show that the specimens retained a high degree of their original biodiversity and, as found across several other marine organisms including shallow water corals [17], reef damselfishes [37], etc., there is a great deal of $\alpha$-diversity variability across specimens belonging to the same taxon. Based on these results, we proceeded with further analysis of the microbial assemblages to gain insight into the structure and function of cold and deep sea water corals from these archival museum specimens.

New insights into the prokaryotic community of cold and deep sea water corals

The coral families investigated here were found to have unique prokaryotic assemblages (Fig. 2b and 2c). At the phylum level, Proteobacteria were recorded at high relative abundance (> 25.2%) in every family investigated, tipping to 89.9% in the Paragorgididae (Fig. 2b; Supp. Table 4). The phyla Verrucomicrobiota, Cyanobacteria, Acidobacteriota and Planctomycetota were also present across multiple coral families but at lower relative abundance (Fig. 2b; Supp. Table 4). At lower taxonomic resolution, the Alphaproteobacteria were the most abundant class in the bacterial communities of each coral family except in the Caryophyllidae (Supp. Table 5). Within the Alphaproteobacteria, sequences associated with members of the orders Rhizobiales, Rhodobacterales and Sphingomonadales were present in every coral family and dominated the prokaryotic communities (Fig. 2c; Supp. Table 6). For instance, 43.6% of the sequences retrieved from the coral family Umbellulidae were associated with the Rhizobiales (Fig. 2c; Supp. Table 6), 41.4% of the sequences retrieved from the coral family
Kophobelemnidae were associated with the Rhodobacterales, and 18.2% of the sequences retrieved from the coral family Keratosidinae were associated with the Sphingomonadales. In contrast to Kellogg et al. [38], which reported a high relative abundance of sequences associated with the genus *Endozoicomonas* in the order Oceanospirillales in the coral genus *Desmophyllum* within the family Caryophilliidae [38], we could only detect a low abundance of sequences associated with this bacterial genus. The specimens collected by Kellogg et al. [38] were from the West Atlantic and ours from the Tasman and Coral seas, therefore it is unclear whether this discrepancy reflects biogeographical patterns affecting the microbiome composition of these corals, or is an artefact of sampling, storage, and handling.

Members of the Sphingomonadales and Rhizobiales are known to play a role in the nitrogen biogeochemical cycle [39]. Evidence shows that shallow water corals rely on microbial partners for nutrient provision [40, 41] and although there is a paucity of information regarding the metabolic interactions between cold and deep sea water corals, and their microbial associates, it is likely that similar processes are also common in this group. Genomic analyses have though shown that Sphingomonadales and Rhizobiales both fix nitrogen in the photic zone [39] and whether they can perform this function in the deep dark ocean is still a mystery, but some heterotrophic Alpha- and Gammaproteobacteria do perform aphotic nitrogen fixation in abyssal waters [42, 43]. The chemical processes by which molecular nitrogen is converted into ammonia without light are not fully understood yet. Nevertheless, it is plausible that the dark and nutrient depleted deep sea ecosystem [1] exerts selective pressure for nitrogen fixing bacteria to establish beneficial associations with corals in these ecosystems.

**Cyanobacteria in the deep**

To our surprise the dataset generated includes 41 ASVs (accounting for 2,500 sequences) belonging to the orders Cyanobacteriales (22), Synechococcales (10), Oxyphotobacteria Incertae Sedis (5) and Obscuribacterales (4; Supp. Table 7). Previous studies investigating cold and deep sea water corals have reported Cyanobacteria associated with their samples. However, they proposed that these microbes were present in the water column and were captured with the corals samples during their retrieval from the deep sea through shallow waters [44]. A further hypothesis to explain the presence of Cyanobacteria in deep sea environments could be their association with sinking particulates that can then be retrieved at great depth [45] and/or caught from the water column at depth by corals. While we do not exclude these hypotheses for our samples, we note that sequences associated with Cyanobacteria were present in every coral family except for the Umbelullidae, ranging from 0.2–38.6% relative abundance (Fig. 2b; Supp. Table 4) and in corals collected in sealed containers at depth by ROV (this precludes the possibility that the corals were bathed in surface waters). Furthermore, studies investigating microbial communities associated with deep-sea foraminifera [46] and rocks [47] have also found an abundance of Cyanobacteria associated with these organisms and proposed that they do not necessarily rely on their photoautotrophic metabolism as their genome shows potential for a hydrogen-based lithoautotrophic metabolism [47]. In fact, Middelburg et al. [12] found evidence of efficient nitrogen recycling in corals at depth and proposed that Cyanobacteria could be involved in this process. Thus, it is possible that the
cyanobacterial sequences retrieved from our specimens could be a ubiquitous component of many cold and deep sea water coral microbial assemblages.

The putative presence of Cyanobacteria in cold and deep sea water corals may not in fact surprising considering that members of this microbial phylum are ubiquitously associated with shallow water corals [18, 40, 48, 49]. Consider also that Cyanobacteria are adapted to environments hostile to organisms thought to exclusively rely on photoautotrophic metabolisms such as caves [50] and the deep seafloor [47]. The presence of Cyanobacteria in ecological niches that do not necessarily meet the physiological requirements of their surficial counterparts suggest that they may have evolved other strategies to survive and grow in the absence of photosynthesis. Cyanobacteria in partnership with cold and deep sea corals may benefit of a stable environment enriched with inorganic compounds such as NO$_3^-$ and SO$_4^{2-}$ that could be used as electron acceptors. Further, interactions with the large microbial community of these corals may also allow for a breadth of molecular handoff to sustain metabolisms optimized to exploit the limited resources of deep sea ecosystem and could in part explain the success of corals in colonizing these ecosystems.

**Mycoplasma in the deep**

Ten unassigned sequences associated with the coral families Keratoisidinae, Kophobelemnidae and Protoptilidae clustered with the bacterial family Mycoplasmataceae in the phylum Mollicutes (Supp. Figure 1). Members of the genus *Mycoplasma* in the family Mycoplasmataceae have been found associated with several deep sea organisms including snails [51], chitons [52], polychaeta [53] and isopods [54], and although their role in these organisms is unclear, it has been speculated they may aid digestion of nutrient-deficient food [51]. Using fluorescent probes, Neulinger et al. [10] identified and described *Candidatus Mycoplasma corallicola*, a proposed species that live on the nematocysts of *Desmophyllum pertusum*. Currently, the role of *Mycoplasma* associated with cold and deep sea water corals is unknown but given their association with nematocysts, Neulinger et al. [10] proposed that these bacteria could be commensal partners that benefit from leakage of hemolymph following perforation of preys by the action of the nematocysts barb. To find support to this hypothesis, we screened 600 *Mycoplasma* genomes recovered from NCBI and found an arsenal of genes encoding transporters of micro-and macro-molecules [e.g. ABC transporters, MFS transporters and ECF transporters; 55, 56, 57]. While these genes are shared across many prokaryotic lineages, their persistence and abundance in bacteria with a reduced genome such as *Mycoplasma* [58] suggest their essential role in scavenging nutrients from the surroundings and in maintaining homeostasis. This finding supports the hypothesis of a commensal lifestyle between families of cold and deep sea water corals and *Mycoplasma* [10]. Furthermore, we also found genes involved in viruses’ immune response [e.g. type IV toxin-antitoxin system AbiEi family antitoxin domain-containing protein and nucleotidyl transferase AbiEii/AbiGii toxin family protein; 59] that are activated by phage infection. Although the main aim of this defence mechanism is probably to protect *Mycoplasma* from viruses’ infections, it is plausible that, as a side effect, it could also aid the host immune system against infection, implying a deeper involvement of *Mycoplasma* in the physiology of coral holobionts. These fascinating and unexpected results highlight
the insights into the functions of cold and deep sea water coral holobionts that can be derived from opportunistic assessments of the microbial assemblages retained in archived museum collections from these largely inaccessible habitats.

**Conclusions**

Our study sheds light on potential new and unique associations of corals in the deep sea with bacterial lineages not previously characterised at the molecular level, which may constitute entirely new prokaryotic lineages. Moreover, our study demonstrates that specimens held in museum collections carry meaningful information about an animals' microbiome, which is detectable through DNA barcoding sequencing. As mentioned, our dataset included sequences from microbes known to be associated with deep water corals [8, 11, 13, 38, 44]. In addition, and surprisingly, we found the Cyanobacteria that could be forming partnerships with corals in the deep sea, as it has with other deep sea organisms and shallow water corals. The deep sea benthic ecosystems are simultaneously the largest and most unknown habitats on earth, which host a breadth of unexplored biodiversity, yet whose remoteness is not remote enough to protect them from the impact of anthropogenic activities. Therefore, we argue that explorations of these remote ecosystems are urgent, with important insights that can be gained through accessing resources within our reach such as specimens held in museum collections.

**Declarations**

**Data availability**


**Acknowledgments**

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**Authors’ contribution**

FR, WL and TA designed the research. FR and MP performed the analyses and created the figures. TB and JH collected a subset of coral specimens used in this project. FR wrote the first draft and edited
subsequent versions. All authors contributed ideas and commented on the text.

References


**Figures**
Figure 1

Map showing the sampling location of the cold and deep sea water corals analysed in this project.

Figure 2
Graphs showing α-diversity indices of the bacterial community across coral families (a), bacterial relative abundance at phylum (b) and order (c) level. Superscript letters indicate coral families belonging to the orders Scleralcyonacea$^a$, Malacalcyonacea$^b$, and Scleractinia$^c$. 