



# Proteomic Profiling of Black Coral (Antipatharia) Skeleton Reveals Hundreds of Skeleton-Associated Proteins Across Two Taxa

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Black corals, ecologically important cnidarians found from shallow to deep ocean depths, form a strong yet flexible skeleton of sclerotized chitin and other biomolecules including proteins. The structure and mechanical properties of the chitin component of the skeleton have been well-characterized. However, the protein component has remained a mystery. Here we used liquid chromatography-tandem mass spectrometry to sequence proteins extracted from two species of common Red Sea black corals following either one or two cleaning steps. We detected hundreds of proteins between the two corals, nearly 70 of which are each other's reciprocal best BLAST hit. Unlike stony corals, only a few of the detected proteins were moderately acidic (biased toward aspartic and/or glutamic acid residues) suggesting less of a role for these types of proteins in black coral skeleton formation as compared to stony corals. No distinct chitin binding domains were found in the proteins, but proteins annotated as having a role in protein and chitin modifications were detected. Our results support the integral role of proteins in black coral skeleton formation, structure, and function.

**Keywords:** antipatharians, chitin, chitinase, skeletal proteins, LC-MS/MS protein sequencing

## INTRODUCTION

Antipatharians (black corals) are colonial cnidarians easily recognized by their black or golden brown chitinous skeletons. They are found from shallow to deep depths and in all oceans as well as the Mediterranean and Red Seas (Opresko, 2001; Wagner et al., 2012; Morgulis et al., 2022), although they face increasing pressure from harvesting (legal and illegal), invasive species, and contaminant introductions (Grigg, 2004; Tordinanahary et al., 2016; Ruiz-Ramos et al., 2017). Their flexible skeletons, typically displaying small protrusions (spines), are covered by animal tissue with each polyp displaying six-fold symmetry (Opresko, 2001). At photic depths, they tend to grow in low light locations and are most frequently observed in locations with strong currents (Wagner et al., 2012; Morgulis et al., 2022).

Black corals are ecologically important as food, habitat/protection, and camouflage for a variety of invertebrates, fish, and mammals, particularly where the corals grow in high density (e.g., Parrish et al., 2002; Boland and Parrish, 2005; De Clippele et al., 2019), although they are not reef builders. Black corals can be home to a high density of organisms; in one study in the Philippines some black

coral forests were associated with over 8,000 individual invertebrates per m<sup>2</sup> (Suarez et al., 2015). Further, in areas with black coral forests, some fish taxa are found only within the colonies (e.g., Boland and Parrish, 2005). However, colonies can be damaged by a variety of human activities including bottom fishing and climate change (Deidun et al., 2015; Godefroid et al., 2022), and at least one species of black coral is considered endangered (IUCN, 2021).

While they have historically been used by humans as money (Tescione, 1973), jewelry (Gress and Andradi-Brown, 2018), and curios (Grigg, 1993), black corals also display a variety of medicinal properties. As suggested by their name (antipathes is Greek for “against diseases”) black corals and their extracts have historically been used in medical treatments including antibacterial activity, reducing fever, pain, and swelling, treating ulcers, and curing lung conditions (Narchi et al., 2015; Peña Moreno, 2017). From a pharmacological perspective, chitin can be deacetylated in alkaline conditions or enzymatically converted to chitosan, a stable non-toxic polymer; this chitosan and its derivatives then displays antibacterial, antifungal, and antiviral properties (review by Nuc and Dobrzycka-Krahel, 2021). Laboratory studies have supported that several of these historical medicinal uses are biochemically founded. For example, mice exposed to cigarette smoke and black coral extract displayed higher superoxide dismutase and malondialdehyde activity and lower lung tissue inflammation than mice exposed to cigarette smoke alone (Bai et al., 2011).

The skeletons of black corals are formed of chitin and other biomolecules (Goldberg, 1976). No minerals are present in the sclerotized skeleton (Goldberg et al., 1994), although the anions iodine and bromine are abundant (Juárez-de la Rosa et al., 2007). Epithelial cells secrete the chitin and other biomolecules that form the extracellular skeleton as concentric layers with a hollow core within the skeleton stalk (Daly et al., 2007). While the chitinous component of the skeleton has been well-characterized in a number of black coral taxa (e.g., Kim et al., 1992; Nowak et al., 2009; Bo et al., 2012; Juárez-de la Rosa et al., 2012), much less is known about the proteinaceous component. Proteins make up around 50% of the skeleton (Goldberg et al., 1994). Glycine, alanine, and histidine are the most abundant amino acids, and treating skeletons with alkaline solutions results in protein loss and shifts in the relative abundance of amino acids (Goldberg et al., 1994).

It has been suggested that chitin provides a viscous-elastic response while the protein component provides a plastic response, yielding the high degree of flexibility of the skeleton (Juárez-de la Rosa et al., 2012). The arrangement of the chitin fibrils in a helical pattern layer by layer may also contribute to the flexibility and reduced buckling under stresses induced by the high water flow environments in which black corals are frequently found (Kim et al., 1992). Further, the spines on the skeleton are cemented to the skeleton creating fixed points to reduce delamination as the skeletons bend and twist in the current (Kim et al., 1992). These mechanical properties may be useful in industry when synthesizing new structural materials. Additional bio-inspiration to be taken from black coral skeleton

could focus on the chitin and protein content of black corals, yielding nanocomposites with industrial and medicinal uses (João et al., 2017; Zheng et al., 2019).

To address the gaps in knowledge about particular biomolecules involved in formation of their chitinous skeleton, we use liquid chromatography-tandem mass spectrometry to characterize the protein composition of ground skeleton from two black coral species, *Antipathes griggi* and *Stichopathes* sp., two commonly occurring black corals in the northern Red Sea (**Figures 1A, B**) (Morgulis et al., 2022). We detected hundreds of proteins in the chitin skeleton of both species. Unlike stony corals, there are few proteins strongly biased toward the acidic amino acids. Gene ontology analysis and domain searching did not reveal any proteins with known chitin binding domains, although several proteins contained an abundance of amino acids associated with chitin binding. Nearly 70 proteins that are each other's reciprocal best BLAST hit were observed in both species, suggesting that, like stony corals, black corals may share a consensus skeletal ‘toolkit’ with a long evolutionary history (Zaquin et al., 2021). Our findings will be important in conservation, pharmaceutical development, and synthesizing materials inspired by strong yet flexible black coral skeletons.

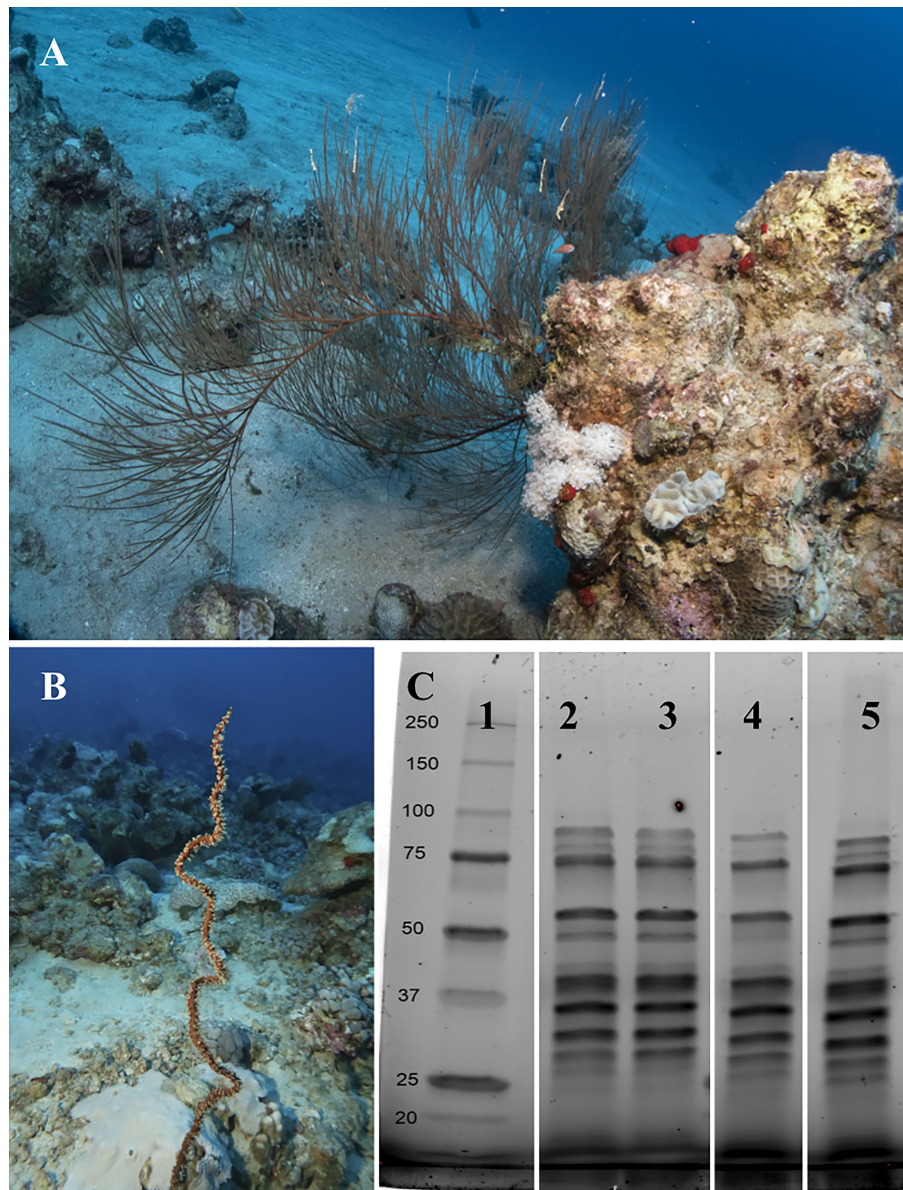
## METHODS

### Specimen Collection

Several specimens of *Antipathes griggi* and *Stichopathes* sp. were collected at 40-60 m depth from the reef adjacent to the Interuniversity Institute in Eilat, Israel under a permit issued by the Israel Nature and Parks Authority (**Figures 1A, B**). Branches or coils of each species were clipped from colonies using underwater shears, segmented into 2-3 cm sections, and immediately transported to the laboratory for preservation in RNA/DNA Shield (Zymo) or 100% ethanol for protein extraction at 4°C. The next day, specimens were transported to the laboratory for processing.

### Samples for Proteomics

Black coral segments of each species were combined and washed copiously in MilliQ water to remove the ethanol, soaked in a 10% bleach solution for 30 minutes at room temperature and for 1 hour with sonication, and then rinsed again with MilliQ and dried at 60°C. The bleaching process was repeated with 50% bleach and the sections were rinsed and re-dried. While the first wash removed most of the tissue, some tentacles of the polyps were still observed at low magnification light microscopy. The second higher concentration bleaching step appeared to remove all tissue by visual inspection. Dried skeletons were ground with a mortar and pestle; *Antipathes* skeletons ground to a finer powder than did *Stichopathes* skeletons. Ground powder was divided; half was used is-as while half was cleaned again in 50% bleach while sonicating for 2 hours, rinsed five times with MilliQ water, and dried at 60°C.



**FIGURE 1** | *Antipathes griggi* (A) and *Stichopathes* sp. (B) collected from the Red Sea adjacent to the Interuniversity Institute in Eilat, Israel, at 40-60 m depth, for proteomic sequencing of the chitinous skeleton. (C) Imaging of SDS-PAGE separation of proteins extracted by chitinase from chitinous black coral skeleton cleaned both before and after grinding. MW marker (lane 1); *Antipathes* cleaned only before grinding (lane 2); *Stichopathes* cleaned only before grinding (lane 3); *Antipathes* cleaned before and after grinding (lane 4); *Stichopathes* cleaned before and after grinding (lane 5). Photos: Hagai Nativ.

## Samples for Transcriptomics

One sample of fresh black coral tissue from each species was flash frozen and then RNA was extracted using the CTAB method (Wang and Stegemann, 2010), followed by use of a Purelink RNA Mini Kit following the manufacturer's instructions. RNA quality was examined by TapeStation (Agilent). Libraries were prepared using the INCPM-mRNA-seq (an internal protocol G-INCPM). The polyA fraction (mRNA) was purified from 500 ng of total RNA followed by fragmentation and generation of double-stranded cDNA. Then, end repair, A base addition,

adaptor ligation and PCR amplification steps were performed. Libraries were evaluated by Qubit (Thermo Fisher Scientific) and TapeStation (Agilent). Sequencing libraries were constructed with barcodes to allow multiplexing of samples. Around 140 million paired-end 150X2-bp reads were sequenced on an Illumina NovaSeq SP300 cyc. Poly-A/T stretches and Illumina adapters were trimmed from the reads using cutadapt; resulting reads shorter than 30 bp were discarded. Trimmed reads were used for assembly using Trinity (Trinity-v2.11.0) and likely bacterial contaminant sequences were filtered out. Highly



similar sequences were clustered with CD-hit (CD-HIT version 4.5.4). Completeness was assessed (BUSCO 4.1.4), with BUSCO scores of 73.8% and 77.2% for *Antipathes* and *Stichopathes*, respectively. Sequence reads were aligned using bowtie2 (version 2.3.4.1), with alignment rates of 97.5% and 95.3% for *Antipathes* and *Stichopathes*, respectively. ORF prediction was performed in TransDecoder-v3.0.1 based on blastp-v2.7.1. Finally, sequences were characterized by InterProScan and annotated in OmicsBox. Transcriptome information and sequences are available in NCBI BioProject PRJNA809900.

## Protein Extraction

Four methods were tested for protein extraction from black coral skeleton powders: Soak in 1 ml each (1) 1 M HCl at room temperature for 1 hour (modified from Shimahara and Takiguchi, 1988); (2) 6 M guanidinium hydrochloride in 8 mM imidazole, 50 mM sodium phosphate monobasic, 50 mM Tris HCl at 30°C for 1 hour (modified from Gildemeister et al., 1994); (3) 50 mM dithiothreitol (DTT) at 100°C for 15 minutes (modified from Mathys et al., 1999); or (4) 1 mg/ml chitinase in PBS 37°C for 2 hours. At the end of the incubation periods all samples were pelleted at 500 xg for 5 minutes at room temperature. Protein concentration of supernatants was quantified by bicinchoninic acid (BCA) assay (**Supplementary Material Table 1**), proteins in 10 µg quantified total protein were visualized by SDS-PAGE separation on Mini-Protean TGX Stain-Free precast gels (BioRad) with 5-minute UV activation, and fractions were stored separately at -80°C until further analysis. Chitinase yielded the darkest bands on UV-activated Stain-Free gels (**Figure 1C**); therefore, only chitinase fractions were submitted for sequencing. HCl also yielded faint bands by SDS-PAGE that were observable after computationally increasing the image contrast (**Supplementary Material Figure 1A**); however, these samples were not submitted for sequencing due to the low protein yield. Guanidinium hydrochloride and DTT extracts did not show any protein bands by SDS-PAGE (**Supplementary Material Figures 1A, B**).

## Protein Sequencing

Proteins extracted by chitinase from ground black coral skeleton cleaned only pre-grinding as well as skeleton cleaned again post-grinding were dissolved in 5% SDS and digested with trypsin using the S-trap method overnight at room temperature. Single samples of each species for each cleaning fraction were processed. The resulting peptides were analyzed using a nanoflow ultra-performance liquid chromatograph (nanoAcquity) coupled to a high resolution, high mass accuracy mass spectrometer (Fusion Lumos) fitted with a Symmetry C18 0.18\*20mm trap column (Waters, Inc) for peptide trapping and then a HSS T3 0.075\*250 mm column (Waters, Inc.) for peptide separation using a gradient of 4-28% (80% acetonitrile, 0.1% Formic acid) for 150 minutes. Spray voltage was set to +2kV. The data were acquired in a Fusion Lumos by a Top Speed Data-Dependent Acquisition method with a cycle time of 3 s. An MS1 scan was performed in the Orbitrap at 120,000 resolution with a maximum injection time of 60 ms and the data were scanned between 300-1800 m/z. MS2

was selected using a monoisotopic precursor selection set to peptides, peptide charge states set to +2-+8 and dynamic exclusion set to 30 s. MS2 was performed using HCD fragmentation scanning in the Orbitrap, with the first mass set to 130 m/z at a resolution of 15,000. Maximum injection time was set to 60 ms with automatic gain control of  $5 \times 10^{-4}$  ions as a fill target. The resulting data were searched against the protein databases derived from our *de novo* black coral transcriptomes described above using the Byonic search engine (Protein Metrics Inc.). A first search was carried out without any false discovery rate (FDR) filtering, to generate a focused database for a second search. The second search was set to 1% FDR, allowing fixed carbamidomethylation on C and variable oxidation on MW, deamidation on NQ and protein N-terminal acetylation. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the Pride partner repository (Perez-Riverol et al., 2019), under the dataset identifier PXD032043.

## Data Sorting

The *de novo* transcriptomes described above for *Antipathes griggi* and *Stichopathes* sp. were used as reference peptide databases for the mass spectrometry analysis. A common contaminants database was also included. Only proteins with at least two significant peptides or at least one significant peptide with at least 10 spectra and an identification score of 250 or greater were retained. To further filter out potential human proteins inadvertently introduced during sample preparation, all sequences were BLASTed against the 'Primates' database in NCBI using Blast2GO 6.0 (Conesa et al., 2005). NCBI-generated sequence alignments of black coral versus Homo sapiens proteins with Blast2GO e-values lower than  $e^{-50}$  and percent mean similarity greater than 50%, e-values lower than  $e^{-100}$ , and percent similarity greater than 80% were removed from our final list of proteins if manual visual examination showed that likely tryptic peptides could have been human, rather than black coral, derived (Peled et al., 2020).

For each taxon, likely coral-specific skeleton proteins were divided into those which were found in skeleton that had only been cleaned pre-grinding (cleaned once), those which were found in skeleton that were cleaned again post-grinding (cleaned twice), and those that were found in both fractions (both). Proteins in each group were then characterized for amino acid composition using SubsetDF in the CHNOSZ package in RStudio (Team, 2019), presence of a signal peptide using the Signal P-5.0 server (Armenteros et al., 2019), and completeness of predicted sequence using the annotations described above. Proteins were further examined for strong bias toward cysteine, aspartic and/or glutamic acid, or tryptophan, lysine, and/or phenylalanine. Domains for completely predicted proteins with a strong amino acid bias were predicted in the PROSITE server (Sigrist et al., 2012) and domain visualizations were created in the PROSITE MyDomains Image Creator (Hulo et al., 2007). Clustering at 30, 50, and 90% sequence similarity of black coral and scleractinian (Drake et al., 2013; Ramos-Silva et al., 2013; Takeuchi et al., 2016; Peled et al., 2020) proteins plus proteins extracted from chitinous cuticles of the mosquito *Anopheles*

*gambiae* (He et al., 2007; Mastrobuoni et al., 2013; Champion et al., 2016; Zhou et al., 2016) was conducted in CD-HIT with default settings (Huang et al., 2010). Gene ontology term enrichment was performed using a two-tailed Enrichment Analysis in Blast2GO with  $p < 0.05$  (Conesa et al., 2005), which integrates the FatiGO package (Al-Shahrour et al., 2004) using the Fisher's Exact Test. Reciprocal BLASTing was performed in the BLAST+ command line application.

## RESULTS AND DISCUSSION

Of the four protein extraction methods tested for the two black coral species - HCl, guanidine hydrochloride, DTT, and chitinase - chitinase yielded the highest amount of protein separated by SDS-PAGE with subsequent imaging (Figure 1C). While BCA assays suggested that HCl, DTT, and guanidine hydrochloride resulted in protein extraction from the chitin skeletons (Supplementary Material Table 1), it appears more likely that these treatments released chitosan, an amide derivative of chitin that can react with the BCA reagents (Kasaai et al., 2013; Gao et al., 2019). Cleaned and uncleaned fractions of black coral skeletal proteins extracted by chitinase were therefore submitted for protein sequencing.

All samples contained at least 100 returned proteins above the designated cutoffs and that were not likely human contaminants (Supplementary Material Table 2). *Antipathes* skeleton yielded 167 proteins that were only in the twice-cleaned fraction, 105 proteins that were only in the once-cleaned fraction, and 99 proteins in both fractions (Figure 2A). From *Stichopathes* skeleton, 93 proteins were only in the twice-cleaned fraction, 62 were in the once-cleaned fraction, and 73 were in both fractions (Figure 2B). Some of the proteins from the singly cleaned fractions were likely not bound to the chitinous skeleton and as such were removed during the second cleaning step as has been shown for removal of non-intracrystalline stony coral skeletal organic matter (Hendy et al., 2012). However, we cannot exclude them from the list of potential proteins involved in the chitinous black coral skeleton as they may still serve a role in skeleton strength as has been shown in squid pen which is also a composite of chitin plus proteins, lipids, and other polysaccharides (Montroni et al., 2021).

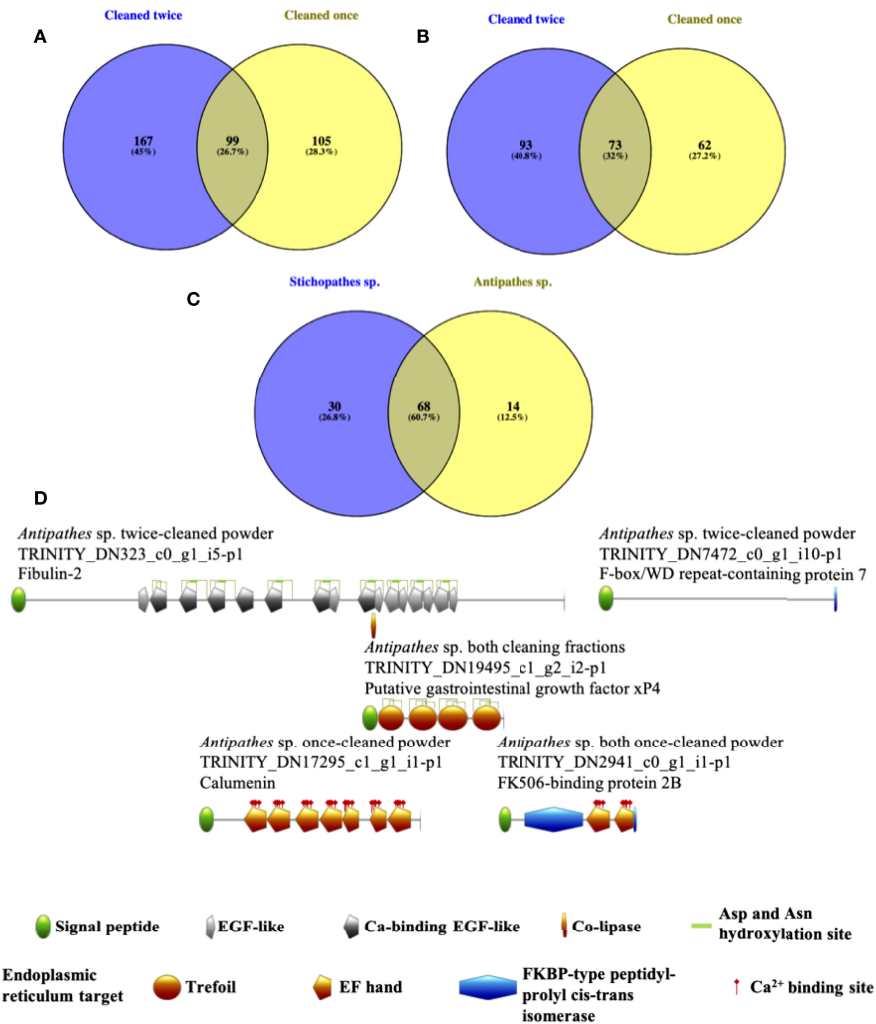
Of the total 371 proteins from *Antipathes* skeleton and 228 proteins from *Stichopathes* skeleton (Additional Files S1 and S2, respectively), 112 were found in the transcriptomes of both species and 68 were each other's reciprocal best BLAST hit (Figure 2C, Supplementary Material Table 3). Distribution of reciprocal best BLAST hit proteins between cleaning fractions between the two taxa is non-random (Fisher's exact test,  $p < 0.05$ , Supplementary Material Table 4). Orthologous proteins found in the same cleaning fractions of both species include a MAM and LDL receptor domain containing protein, three coadhesin/hemicentin/mucin proteins, and four collagen alpha proteins among others. Interestingly, these protein types are also known from stony coral skeleton, as are several protein types that were detected in both black coral skeletons but in different cleaning

fractions including an EGF and laminin G domain-containing protein, a potential protocadherin, and uncharacterized skeletal organic matrix protein 5 (Drake et al., 2013; Ramos-Silva et al., 2013; Takeuchi et al., 2016; Peled et al., 2020). Only *Antipathes* skeleton contained a carbonic anhydrase, which is known from stony coral skeleton from multiple species (Drake et al., 2013; Ramos-Silva et al., 2013; Peled et al., 2020), where it likely supplies bicarbonate for the eventual production of the calcium carbonate exoskeleton of scleractinians (Bertucci et al., 2013; Comeau et al., 2017).

Not all proteins were completely predicted (Supplementary Material Table 2). Of 84 proteins in the twice-cleaned fraction extracted from *Antipathes* that were completely predicted, two displayed a disproportionate amount of aspartic and glutamic acids ('disproportionate' defined as  $>20\%$  of total amino acids) and four had  $>10\%$  cysteine; three of those with excess cysteine had signal peptides. Nearly half the number of completely predicted proteins (47) were found only in the once-cleaned fraction from *Antipathes*, and 20 of these displayed a signal peptide with two of this subset also showing excess aspartic and glutamic acids. Eighteen proteins observed in both fractions were both completely predicted and displayed a signal peptide; two of these were  $>10\%$  cysteine.

Fewer proteins were completely predicted in *Stichopathes* (Supplementary Material Table 2). In the twice-cleaned fraction for this taxon, 16 of 42 completely predicted proteins have signal peptides and only one is  $>10\%$  C. Similarly, 23 of 44 complete proteins found in both fractions have signal peptides and only one is  $>10\%$  cysteine. No completely predicted proteins in these two fractions were biased toward acidic amino acids. The once-cleaned fraction also showed a similar breakdown of completeness and signal peptides, with 34 proteins being complete and 12 of these having a signal peptide; however, none of these were biased toward any amino acids of interest.

Bias of skeletal proteins toward acidic amino acids (aspartic and glutamic acids) is well-documented in organisms that produce biocarbonates (e.g., Nocente-McGrath et al., 1989; Gotliv et al., 2005; Mass et al., 2013) and biophosphates (He et al., 2003), and even in collagen-dominated biophosphates there are several known acidic non-collagenous proteins (Gorski, 2011). Unlike either stony coral skeletons or vertebrate bone, however, black corals' skeletons are constructed of sclerotized chitin, a long-chained polysaccharide (Goldberg, 1976). Chitin displays a similar hierarchical organization to biocarbonates (Ehrlich, 2010), and acidic chitinases (e.g., Samac et al., 1990) have been characterized from a number of organisms. Further, an acidic chitin binding protein appears to be involved in the precipitation of calcium phosphate and then calcium carbonate in crustacean teeth (Tynyakov et al., 2015). Our results suggest that acidic proteins may be involved in black coral chitinous skeleton development; however, despite likely sharing conserved biomineralization-related genes with stony corals (review by Gilbert et al., 2022), black coral acidic proteins are perhaps not as central to the process as they appear to be in allowing stony corals



**FIGURE 2** | For both *Antipathes* (A) and *Stichopathes* (B) we observed ~30% overlap in protein detection between skeleton cleaned only before grinding (yellow) and skeleton clean both before and after grinding (purple). Unique proteins were also found in both fractions. Further, for both species, the largest number of proteins were found in the twice-cleaned fractions (purple, a & b). 60.7% of detected proteins with reciprocal best BLAST hits between the two species were observed in both species (C) across the various cleaning fractions. (D) Predicted domains in proteins extracted from black coral skeleton using chitinase. Only proteins that were completely predicted, display a proposed signal peptide, and are biased toward key amino acids were considered. Although several *Stichopathes* skeletal proteins meet these criteria, none are predicted to have any domains.

biomineralize (Mass et al., 2013) and to counter the effects of ocean acidification (e.g., Drake et al., 2017).

Several amino acids are associated with chitin binding domains, including cysteine, phenylalanine, tryptophan, and tyrosine (Shen and Jacobs-Lorena, 1999). Invertebrate and plant chitin binding proteins typically display several conserved cysteines that are likely involved in disulfide bonds related to protein secondary and tertiary structure (Shen and Jacobs-Lorena, 1999). Tyrosine and phenylalanine are conserved components of the R&R consensus motif of chitin binding cuticle proteins (Rebers and Willis, 2001), and tyrosine and tryptophan are crucial conserved amino acids in the binding cleft of *Bacillus* chitinase. Despite a small number of completely predicted detected proteins being biased toward cysteine, and

none toward phenylalanine, tryptophan, or tyrosine, some of the proteins may have chitin binding capabilities and should be tested in the future.

We also considered only those full proteins that are proposed to have a signal peptide for export to the cell membrane or extracellular space (review by von Heijne, 1990), and then queried this final group for those with predicted domains. Out of nine proteins between the two species that meet the above criteria, five are predicted to have known domains, although none are explicitly chitin binding, and no such proteins from *Stichopathes* were found to have predicted domains (Figure 2D). A protein annotated as fibulin-2 contains several EGF-like domains, some of which can be Ca<sup>2+</sup> binding. EGF-like domains are found in extracellular matrix proteins and can be

**TABLE 1 |** Black coral, stony coral, and mosquito exoskeleton proteins clustered by sequence similarity in CD-Hit.

<b>50% Similarity</b>				
<b>Cluster Number</b>	<b>Annotation</b>	<b>Sequence ID</b>	<b>% Sequence similarity</b>	
1	MAM and LDL-receptor class A domain-containing protein 2-like	XP_022794736.1		
	MAM and LDL-receptor class A domain-containing protein 2	Antipathes_TRINITY_DN90165_c0_g1_i1.p1	64.3%	
	MAM and LDL-receptor class A domain-containing protein 2-like	Stichopathes_TRINITY_DN24487_c0_g1_TRINITY_DN24487_c0_g1_i1_g.57476	64.6%	
	MAM and LDL-receptor class A domain-containing protein 2-like	Stichopathes_TRINITY_DN45621_c1_g1_TRINITY_DN45621_c1_g1_i1_g.64725	62.6%	
	MAM and LDL-receptor class A domain-containing protein 2-like	Stichopathes_TRINITY_DN6745_c0_g1_TRINITY_DN6745_c0_g1_i1_g.29670	64.4%	
	MAM and LDL-receptor class A domain-containing protein 2-like	Stichopathes_TRINITY_DN8568_c0_g1_TRINITY_DN8568_c0_g1_i3_g.28233	71.6%	
	MAM and LDL-receptor class A domain-containing protein 2-like	JT011118.1	55.9%	
	MAM and LDL-receptor domain- containing protein 2	JR994474.1	50.8%	
	MAM and LDL-receptor class A domain-containing protein 2-like	g6066	56.4%	
	MAM and LDL-receptor class A domain-containing protein 2-like	g1714	72.0%	
	MAM and LDL-receptor class A domain-containing protein 2-like	g13890	96.3%	
	MAM and LDL-receptor class A domain-containing protein 2-like	g1647	55.5%	
	MAM and LDL-receptor class A domain-containing protein 2-like	g15955	50.1%	
	2	MAM and LDL-receptor class A domain-containing protein 2	Stichopathes_TRINITY_DN1603_c0_g1_TRINITY_DN1603_c0_g1_i7_g.121738	
		MAM and LDL-receptor class A domain-containing protein 2	Antipathes_TRINITY_DN10485_c0_g1_i1.p1	96.7%
MAM and LDL-receptor class A domain-containing protein 2		Antipathes_TRINITY_DN1203_c0_g1_i1.p1	96.7%	
MAM and LDL-receptor class A domain-containing protein 2-like		aug_v2a.09969.t1	59.6%	
3	protein lingerer-like	XP_022806664.1		
	NA	Antipathes_TRINITY_DN2417_c0_g1_i1.p1	53.3%	
4	collagen alpha-1(II) chain-like	Stichopathes_TRINITY_DN47849_c2_g1_TRINITY_DN47849_c2_g1_i1_g.74739	53.3%	
	collagen alpha-2(V) chain-like	Stichopathes_TRINITY_DN7187_c0_g1_TRINITY_DN7187_c0_g1_i4_g.116238		
5	NA	Antipathes_TRINITY_DN60847_c0_g1_i2.p1	90.7%	
	collagen alpha chain-like	JR991083.1	54.7%	
6	MAM and LDL-receptor class A domain-containing protein 1-like isoform X1	Stichopathes_TRINITY_DN26_c0_g1_TRINITY_DN26_c0_g1_i3_g.3085		
	CUB and peptidase domain-containing protein 1 {ECO:0000303 PubMed:23765379}	Antipathes_TRINITY_DN6910_c0_g1_i2.p1	76.9%	
7	CUB and peptidase domain-containing protein 2-like	XP_022780694.1	50.8%	
	MAM and LDL-receptor class A domain-containing protein 2-like	aug_v2a.09968.t1		
8	MAM and LDL-receptor class A domain-containing protein 1	Antipathes_TRINITY_DN5167_c0_g1_i1.p1	63.3%	
	NA	Antipathes_TRINITY_DN20198_c0_g1_i1.p1		
9	uncharacterized	g8985	72.4%	
<b>30% Similarity</b>				
<b>Cluster Number</b>	<b>Annotation</b>	<b>Sequence ID</b>	<b>% Sequence similarity</b>	
1	MAM and LDL-receptor class A domain-containing protein 2-like	XP_022794736.1		
	MAM and LDL-receptor class A domain-containing protein 2-like	JR994474.1	61.4%	
	MAM and LDL-receptor class A domain-containing protein 2-like	JT011118.1	62.3%	
	MAM and LDL-receptor class A domain-containing protein 2-like	aug_v2a.09968.t1	50.3%	

*(Continued)*

TABLE 1 | Continued

## 30% Similarity

Cluster Number	Annotation	Sequence ID	% Sequence similarity
	MAM and LDL-receptor class A domain-containing protein 2	Stichopathes_TRINITY_DN1603_c0_g1_TRINITY_DN1603_c0_g1_i7_g.121738	37.7%
	MAM and LDL-receptor class A domain-containing protein 2-like	aug_v2a.09969.t1	38.1%
	MAM and LDL-receptor class A domain-containing protein 2-like	g15955	65.4%
	MAM and LDL-receptor class A domain-containing protein 2-like	g1647	84.8%
	MAM and LDL-receptor class A domain-containing protein 2-like	Stichopathes_TRINITY_DN8568_c0_g1_TRINITY_DN8568_c0_g1_i3_g.28233	71.2%
	MAM and LDL-receptor class A domain-containing protein 2-like	Stichopathes_TRINITY_DN6745_c0_g1_TRINITY_DN6745_c0_g1_i1_g.29670	63.0%
	MAM and LDL-receptor class A domain-containing protein 2-like	Stichopathes_TRINITY_DN24487_c0_g1_TRINITY_DN24487_c0_g1_i1_g.57476	64.1%
	MAM and LDL-receptor class A domain-containing protein 2-like	g1714	95.5%
	MAM and LDL-receptor class A domain-containing protein 2-like	g6066	71.4%
	MAM and LDL-receptor class A domain-containing protein 1	Antipathes_TRINITY_DN5167_c0_g1_i1.p1	58.2%
	MAM and LDL-receptor class A domain-containing protein 2-like	g13890	95.9%
	MAM and LDL-receptor class A domain-containing protein 2	Antipathes_TRINITY_DN10485_c0_g1_i1.p1	62.7%
	MAM and LDL-receptor class A domain-containing protein 2-like	Stichopathes_TRINITY_DN45621_c1_g1_TRINITY_DN45621_c1_g1_i1_g.64725	62.6%
	MAM and LDL-receptor class A domain-containing protein 2	Antipathes_TRINITY_DN90165_c0_g1_i1.p1	63.6%
2	EGF and laminin G domain-containing protein-like	XP_022804012.1	
	EGF and laminin G domain-containing protein-like	Stichopathes_TRINITY_DN2042_c0_g1_TRINITY_DN2042_c0_g1_i2_g.8370	41.3%
	EGF and laminin G domain-containing protein-like	JR980881.1	47.4%
	EGF and laminin G domain-containing protein-like	aug_v2a.06122.t1	43.1%
	EGF and laminin G domain-containing protein	Antipathes_TRINITY_DN21208_c0_g1_i1.p1	43.9%
	EGF and laminin G domain-containing protein-like	g7086	86.2%
	EGF and laminin G domain-containing protein-like	g34749	85.1%
	EGF and laminin G domain-containing protein-like	g20420	82.3%
	EGF and laminin G domain-containing protein-like	g20041	82.9%
3	MAM and LDL-receptor class A domain-containing protein 1-like isoform X1	Stichopathes_TRINITY_DN26_c0_g1_TRINITY_DN26_c0_g1_i14_g.3080	
	MAM and LDL-receptor class A domain-containing protein 2	Antipathes_TRINITY_DN507_c0_g1_i17.p1	52.1%
	MAM and LDL-receptor class A domain-containing protein 1-like isoform X1	Stichopathes_TRINITY_DN26_c0_g1_TRINITY_DN26_c0_g1_i3_g.3085	55.0%
	MAM and LDL-receptor class A domain-containing protein 1-like isoform X1	Stichopathes_TRINITY_DN26_c0_g1_TRINITY_DN26_c0_g1_i4_g.3083	73.6%
	Zinc metalloproteinase nas-15	Antipathes_TRINITY_DN385_c0_g1_i12.p1	53.6%
	mepirin A subunit beta-like	Stichopathes_TRINITY_DN3619_c0_g1_TRINITY_DN3619_c0_g1_i1_g.25688	41.7%
	CUB and peptidase domain-containing protein 1	Antipathes_TRINITY_DN6910_c0_g1_i2.p1	66.0%
	CUB and peptidase domain-containing protein 2-like	XP_022780694.1	47.9%
4	ZP domain-containing protein-like	JN631095.1	
	ZP domain-containing protein-like	XP_022806326.1	57.9%
	ZP domain-containing protein-like	aug_v2a.07627.t1	91.9%
	ZP domain-containing protein	Antipathes_TRINITY_DN41751_c1_g1_i1.p1	47.1%
	ZP domain-containing protein-like	g907	62.2%
	ZP domain-containing protein-like	g18277	62.1%
5	fibronectin type III domain-containing protein-like	JR993827.1	
	fibronectin type III domain-containing protein-like	Stichopathes_TRINITY_DN1772_c0_g1_TRINITY_DN1772_c0_g1_i9_g.72338	43.8%
	Fibronectin type III domain-containing protein	Antipathes_TRINITY_DN1513_c0_g1_i5.p1	47.2%
	fibronectin type III domain-containing protein-like	g22569	49.6%
	Fibronectin type III domain-containing protein	Antipathes_TRINITY_DN1513_c0_g1_i7.p1	48.7%

(Continued)



TABLE 1 | Continued

30% Similarity			
Cluster Number	Annotation	Sequence ID	% Sequence similarity
6	Uncharacterized skeletal organic matrix protein 5	Antipathes_TRINITY_DN6046_c0_g1_i1.p1	
	uncharacterized skeletal organic matrix protein 5-like	Stichopathes_TRINITY_DN648_c1_g1_TRINITY_DN648_c1_g1_i10_g.36676	73.9%
	uncharacterized skeletal organic matrix protein 5-like	JR973117.1	36.3%
	uncharacterized skeletal organic matrix protein 5-like	aug_v2a.22918.t1	35.5%
7	NA	Stichopathes_TRINITY_DN4778_c0_g2_TRINITY_DN4778_c0_g2_i1_g.1379	79.2%
	golgin subfamily A member 4-like	Antipathes_TRINITY_DN3769_c0_g1_i7.p1	
	protein MLP1-like	Stichopathes_TRINITY_DN2366_c0_g1_TRINITY_DN2366_c0_g1_i2_g.62183	97.8%
8	Matrix metalloproteinase-24	g9861	48.5%
	matrix metalloproteinase-25-like	Antipathes_TRINITY_DN2668_c0_g2_i1.p1	
9	collagenase 3-like	Stichopathes_TRINITY_DN70_c0_g1_TRINITY_DN70_c0_g1_i3_g.3376	91.3%
	kielin/chordin-like protein	XP_022783952.1	32.5%
10	Fibroblast growth factor 20	g5540	
	kielin/chordin-like protein	Antipathes_TRINITY_DN22734_c0_g1_i4.p1	32.9%
11	NA	g39770	57.1%
	uncharacterized	Antipathes_TRINITY_DN20198_c0_g1_i1.p1	
11	digestive cysteine proteinase 1-like	g8985	72.4%
	Cathepsin L	XP_022803524.1	
		Antipathes_TRINITY_DN1770_c0_g1_i11.p1	35.3%

While sequence similarity was observed between black and stony corals at 30% and 50% similarity, none was observed between any corals and mosquitos.

involved in protein-protein interactions (review by Engel, 1989; Rao et al., 1995). Trefoil domains have also been found in extracellular matrix proteins; the domain typically contains six cysteines that form disulfide bridges; these proteins may interact with mucin (Thim, 1997), which has been found in the skeleton-associated extracellular matrix of stony corals (Ramos-Silva et al., 2013; Takeuchi et al., 2016; Peled et al., 2020) and which associates with chitin in arthropod guts (Dias et al., 2018), although mucin has not been sequenced from arthropod cuticle.

To determine if invertebrates that produce a chitinous skeleton display skeletal proteome similarities across divergent

phyla, we compared the black coral skeletal proteome with that of mosquito cuticle. No clustering of black coral and *Anopheles gambiae* cuticular proteins was observed at 30, 50, or 90% sequence similarity. While no clustering of black and stony coral skeletal proteins was observed at 90% similarity, several clusters were observed at 50 (7 clusters) and 30% (11 clusters) similarity. Clustered black and stony coral proteins are annotated as collagenase like, CUB and peptidase domain containing, digestive cysteine proteins like, EGF and laminin G domain containing, fibronectin type III domain containing, kielin/chordin like, MAM and LDL receptor domain containing, protein MLP like, uncharacterized skeletal organic matrix

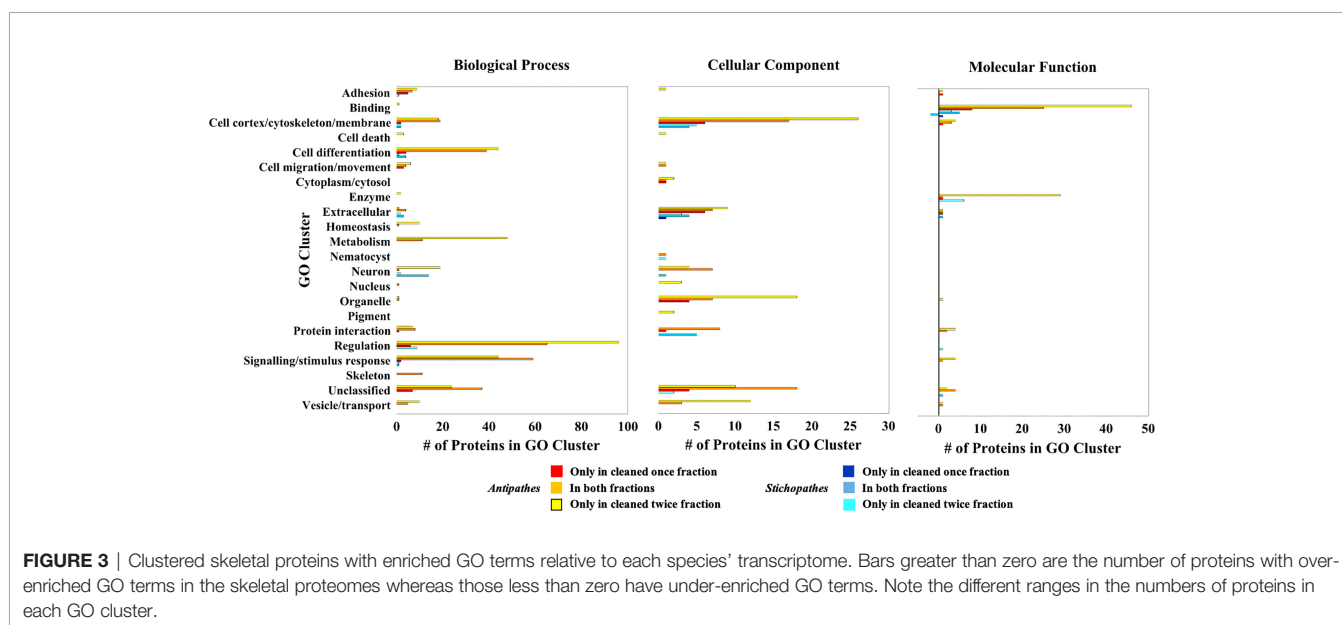


FIGURE 3 | Clustered skeletal proteins with enriched GO terms relative to each species' transcriptome. Bars greater than zero are the number of proteins with over-enriched GO terms in the skeletal proteomes whereas those less than zero have under-enriched GO terms. Note the different ranges in the numbers of proteins in each GO cluster.

protein 5, ZP domain containing, and uncharacterized in stony corals (Table 1).

*Antipathes* skeletal proteins sequenced in this work are over-enriched in GO terms related to binding, the cell cortex/cytoskeleton/membrane, cell differentiation, enzymes, extracellular location, metabolism, organelles, regulation, signaling/stimulus response, and vesicles/transport (Figure 3). Similarly, *Stichopathes* skeletal proteins are over-enriched in GO terms for binding, enzymes, extracellular location, and regulation; additionally, they are enriched for neurons and protein interaction (Figure 3). The only GO term cluster that was under-enriched was observed for *Stichopathes* and included terms related to binding: organic cyclic compound binding [GO:0097159] and heterocyclic compound binding [GO:1901363]. Proteins with extracellular location and membrane GO terms may be important as black coral chitin formation and sclerotization are extracellular processes (Wagner et al., 2012). As vesicular transport of ions and organic molecules that form the skeleton appears to be important in stony corals (Schmidt et al., 2022), vesicle and transport proteins may also play a role in black coral skeleton formation. Proteins which function as enzymes may also be important to the formation and modification of the chitinous skeleton; these include a carbonic anhydrase in *Antipathes*, a calmodulin in *Stichopathes*, and several proteases and chitinases in both species.

## CONCLUSIONS

While the structure and mechanical properties of black coral skeleton chitin have been well-characterized, to date, their protein composition has received less attention. To address this research gap, we sequenced the skeletal proteome of two species of common Red Sea black corals under two steps of strong oxidative cleaning. Our analyses reveal hundreds of proteins in the skeletons of each species, with ~70 being each other's reciprocal best BLAST hit, potentially representing a conserved toolkit. A small number of proteins can be considered moderately acidic and only a few are biased toward amino acids known to be involved in chitin binding. Instead, our GO analysis shows that terms related to vesicles and transport, membrane structure, and enzymatic functionality are enriched. This initial work suggests that more research is required to fully comprehend the functions of black coral skeletal proteins and their interactions with chitin.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the following online repositories: (1) Transcriptome data are available under

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- NCBI BioProject PRJNA809900. (2) The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al.) partner repository with the dataset identifier PXD032043 and 10.6019/PXD032043.

## AUTHOR CONTRIBUTIONS

Conceptualization, investigation, writing—original draft preparation, JD and TM. Formal analysis, data curation, visualization, JD. Resources, supervision, funding acquisition, TM. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.904835/full#supplementary-material>

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