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Environmental DNA Reveals Diverse and Depth-Stratified Biodiversity in East Indian Ocean Submarine Canyons

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ABSTRACT

Submarine canyons are globally recognized as biodiversity hotspots, yet logistical challenges in accessing deep-sea environments hinder comprehensive surveys of their biota. This study presents the first extensive environmental DNA (eDNA) survey of the Cape Range and Cloates submarine canyons in the East Indian Ocean, integrating eDNA metabarcoding, remotely operated vehicle (ROV) imagery, and specimen collection to develop curated DNA reference sequences. Two metabarcoding assays (COI Leray and 16S Fish) were applied to 178 ten-liter water samples collected across 5 depths: surface, 200, 500, 1000 m, and bottom (1750–4540 m). These assays detected 226 species spanning 126 families, with each canyon revealing unique species. Notably, our study identified 83 putative new records or range extensions, including a range extension for the sleeper shark (*Somniosus*) and the elusive giant squid (*Architeuthis dux*), and the first Western Australian record of the faceless cusk eel (*Typhlonus nasus*). We also detected deep-diving mammals, including the pygmy sperm whale (*Kogia breviceps*) and Cuvier's beaked whale (*Ziphius cavirostris*). Our results revealed strong assay-specific differences in vertical stratification patterns, with clear depth-related signals in the COI Leray dataset and weaker, broad-scale separation in the 16S Fish dataset. These disparities likely reflect the biological characteristics of the target taxa for each assay, as well as the dynamic nature of submarine canyons influencing vertical mixing. Together, these findings highlight the complexity of interpreting spatial biodiversity patterns using eDNA and the necessity of considering biological, ecological, and physical context of both target taxa and environment in future studies. Our work demonstrates the efficacy of eDNA metabarcoding in exploring submarine canyon biodiversity and its potential for characterizing biodiversity in unexplored deep-sea habitats. The implementation of eDNA metabarcoding in deep-sea research shows promise as a tool for establishing ecological baselines and informing conservation practices as growing anthropogenic pressures threaten these unique ecosystems.

1 | Introduction

Submarine canyons are major geographical features of continental shelf margins (Harris et al. 2014), connecting relatively shallow productive shelves to deep-ocean basins (Puig

et al. 2014). They act as conduits for the transportation of organic matter and macrophytes between the continental shelf and abyssal depths (Harris and Whiteway 2011; Vetter and Dayton 1999). The concentration of organic matter enhances local primary productivity in these canyons (De Leo et al. 2010;

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Vetter et al. 2010), providing key feeding, spawning, and recruitment grounds for both commercially important and charismatic megafauna species (Ramírez-Llodra et al. 2012; D'Onghia et al. 2015; Farrugio 2012; Fernandez-Arcaya et al. 2013; Moors-Murphy 2014; Yoklavich et al. 2000). The complex and abrupt topography of these canyons creates unique habitats that disrupt expected diversity patterns (Levin et al. 2010; Puig et al. 2014), leading to locally distinct assemblages, high rates of endemism, and potential speciation through physical isolation (Cunha et al. 2011; Gunton et al. 2015; Levin et al. 2001; Ramírez-Llodra, Company, et al. 2010; Schlacher et al. 2007). As a result, these environments are largely considered to be biodiversity hotspots (De Leo et al. 2010; Robertson et al. 2020; Vetter et al. 2010) that provide important refuge areas for many rare and threatened species (Bianchelli et al. 2010; Fabri et al. 2014; Huvenne et al. 2011).

Although submarine canyons are widespread along continental margins, patterns in community composition and species richness have only been investigated in a relatively small subset of submarine canyons (De Leo and Puig 2018; Matos et al. 2018). Many remain largely unexplored with many undescribed species (Matos et al. 2018) due to the inherent difficulties associated with accessing and sampling these environments (Ramírez-Llodra, Brandt, et al. 2010). The deep sea is immense and remote, and comprehensive surveys of this environment are expensive, and limited in time and space (Duhamet et al. 2023; Paulus 2021; Ramírez-Llodra, Brandt, et al. 2010). Additionally, the majority of baseline biodiversity data is obtained through benthic sampling techniques, with relatively limited attention given to pelagic biodiversity in these ecosystems (St. John et al. 2016; Webb et al. 2010), primarily due to the difficulties posed by the vast expanse of the deep pelagic environment (Ramírez-Llodra, Brandt, et al. 2010). Despite these difficulties, these submarine canyons are increasingly threatened by direct and indirect anthropogenic pressures from fisheries, climate change, pollution, and extraction or mining (Daly et al. 2018; Fabri et al. 2014; Jamieson et al. 2019; Orejas et al. 2009; Trotter et al. 2019). As a result, these ecosystems may experience a silent erosion of deep benthic and pelagic biota, with many species driven to extinction before they are discovered.

Biological surveys of submarine canyons are logistically and financially constrained by the challenges of comprehensively sampling across broad spatial scales (over large distances and to great depths) in often remote locations (McClain and Barry 2010; Moors-Murphy 2014). Although trawling has been a popular approach for surveying deep-sea ecosystems (Nóbrega et al. 2023; Orlov and Volvenko 2022; Sánchez et al. 2008), it is not feasible across areas of unsuitable substrates, such as steep canyon walls, rocky outcrops, hard substrates, and complex high-relief terrain (Koslow et al. 2000). Moreover, trawling should be avoided in unexplored regions given the substantial and often irreversible effects it can have on these vulnerable ecosystems (Good et al. 2022; Puig et al. 2012). For example, a study in the north-western Mediterranean Sea examined the effects of daily bottom trawling for deep-sea shrimp in a submarine canyon and found significant declines in organic matter content (up to 52%), slower carbon turnover (around 37%), decreased meiofauna abundance (80%), and lower biodiversity (50%) in trawled areas compared to untrawled areas (Pusceddu et al. 2014).

Visual surveys conducted using remotely operated underwater vehicles (ROV) are a non-invasive alternative that have frequently been used for biodiversity surveys in deep-sea environments (Ayma et al. 2016; de Mendonça and Metaxas 2021; Dumke et al. 2018; Marsh et al. 2012) and provide a more targeted approach than “blind” trawl or grab survey methods (Przeslawski et al. 2018). However, the use of equipment like ROVs can easily introduce sampling variability and bias through sampling design (e.g., opportunistic video vs. imagery transects; Duffy et al. 2014) and by affecting the behavior of deep-sea fauna (Ayma et al. 2016; Lorange and Trenkel 2006). Additionally, survey time is generally limited in deep-sea environments, and more cryptic species may not be detected in visual surveys (Sward et al. 2019). It is challenging to confidently identify organisms to species-level from video transects in the deep-sea, hence the development of surveys using unmanned vehicles driven by ship-based pilots with advanced specimen collection skills. The collection of specimens enables taxonomic identifications to be verified using morphological and molecular means and greatly increases confidence in biodiversity studies (Baco and Cairns 2012; Bode et al. 2017). It also allows the subsequent description of those species if new to science.

The use of contemporary genetic techniques, such as environmental DNA (eDNA) metabarcoding, has become increasingly common in biomonitoring and is now at the forefront of biodiversity research (Takahashi et al. 2023). eDNA metabarcoding describes patterns in biodiversity through the amplification of trace amounts of DNA naturally shed into the environment by organisms, profiling a wide range of biota without the need for visual observation. Surveys using eDNA have demonstrated increased sensitivity to the presence of rare and endangered species in comparison to conventional survey methods (Nester et al. 2023), expanding on total observed diversity. Importantly, it has been successfully used in deep-sea environments to describe communities across both spatial scales (Guardiola et al. 2016; Laroche et al. 2020; Lejzerowicz et al. 2014) and depth gradients (Canals et al. 2021; Feng et al. 2022), overcoming the difficulties associated with sampling these environments and expanding the breadth of biodiversity detected.

The Cape Range and Cloates canyons are two large submarine canyons on the north-western margin of Australia, reaching depths of 4900 and 4400 m respectively (Post et al. 2022). These canyons are connected to the continental shelf via channels, connecting deep abyssal waters to shallow waters adjacent to the highly biodiverse, World Heritage-listed Ningaloo Reef (Post et al. 2022). The heads of these canyons are associated with localized areas of high productivity due to the interaction of upwelling nutrient-rich Antarctic Intermediate Water with the Leeuwin Current (Brewer et al. 2007; Lowe et al. 2012). The canyons are recognized as key ecological features within the Gascoyne Marine Park (Falkner et al. 2009), but to date remain biologically unexplored. An improved understanding of these ecosystems will facilitate effective management of the canyons as part of the Gascoyne Marine Park network (Bond and Jamieson 2022) and inform conservation actions in the face of increasing oil and gas initiatives.

Here, we report on the first biological survey of the deep-sea Cape Range and Cloates Canyons in the East Indian Ocean

(FK200308) using a combination of eDNA metabarcoding and Remote Operated Vehicle (ROV) underwater imagery and specimen collection. As eDNA approaches rely heavily on comprehensive genetic reference material, which is lacking for many remote or unexplored locations, we created a custom reference database using museum-vouchered specimens collected from the canyons via the ROV. Our study uses eDNA metabarcoding to: (1) assess variation in diversity and community composition between Cape Range and Cloates canyons, and along a depth gradient (surface, 200, 500, 1000 m, “bottom”); (2) uncover potential new species records and range extensions; and (3) compare diversity patterns and taxonomic resolution captured by eDNA and ROV sampling approaches carried out simultaneously. eDNA technologies are yet to be widely applied in Australia's deep-sea environments, and the limited availability of reference data highlights both the challenges and opportunities of characterizing previously unsampled biodiversity. By characterizing the biodiversity of the Cape Range and Cloates canyons, we will generate important biological baseline information that can provide a foundation for future management and conservation efforts.

2 | Methods

2.1 | ROV Imagery and Specimen Collection

ROV dives were completed across 16 sites in the Cape Range ($n=12$) and Cloates ($n=4$) canyons in March–April 2020 (FK200308; Figure 1; Table S1). Poor weather and limited crew due to the emerging COVID-19 pandemic reduced the number of sites in the Cloates Canyon. Video transects and specimen collection were completed on board the RV *Falkor* using the ROV *SuBastian*, which operates at depths up to 4500 m (Schmidt Ocean Institute 2019). It is equipped with a Sulis Subsea Z70 deep-sea science camera, with 4K optics and sensors for temperature, depth, conductivity and oxygen (Schmidt Ocean Institute 2019). A robotic arm, suction sampler, nets and

scoops were used to collect biological specimens and place them into multi-chamber insulated bioboxes or chambers. Specimens were photographed, subsampled for tissue, and preserved appropriately to retain morphological characters. All specimens were accessioned into the Collections & Research Centre of the Western Australian Museum (Welshpool, Perth, Western Australia). Identifications were made by taxonomists participating onboard or by collaborating scientists.

Survey sites were selected by generating random points along predefined depth contours within the multibeam-mapped extent of each canyon. Points that fell away from canyon walls or outside ROV-operationally accessible terrain were repositioned to the nearest canyon wall to ensure sampling targeted steep canyon habitats and fully utilized the ROV's capability to traverse complex terrain. Twelve quantitative visual transects were surveyed in Cape Range Canyon following Australian national ROV survey protocols (Monk et al. 2024). Quantitative transects were run for 500 m upslope, at approximately 2 m from the seafloor or rock wall at a speed of 0.3 knots (Post et al. 2022). Transect images were annotated using SQUIDLE+ (<http://squidle.org/>) from images acquired every 10–20 s to ensure continuous imagery with minimal spatial overlap. Biota and substrates were characterized with the national standard operating procedures established for marine imagery analysis (Carroll et al. 2018), using the CATAMI image classification scheme (Collaborative and Automated Tools for Analysis of Marine Imagery; Althaus et al. 2015; Table S2). In addition to the quantitative 500-m transects, qualitative meandering transects were also undertaken from all 16 sites in which still images were acquired of organisms of interest. Specimens collected were identified to the lowest taxonomic level by participating taxonomists aboard the RV *Falkor* or at the WAM Collections and Research Centre (Welshpool, Western Australia). CTD sampling was conducted at the same sites as ROV deployments, with casts positioned to overlap ROV transects and as such, CTD sites followed the same site selection framework and did not constitute independent sampling locations.

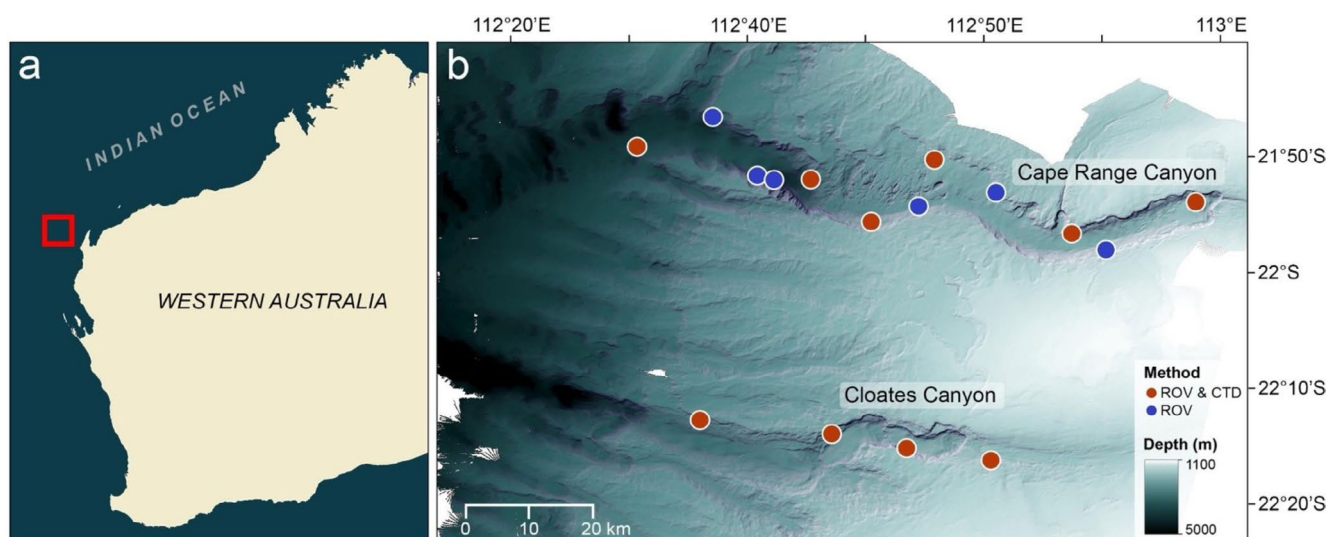


FIGURE 1 | Locations of all ROV transects and CTD casts in the Cape Range and Cloates canyons, Western Australia, underlaid by high-resolution bathymetry collected during the survey. (a) Location of the Cape Range and Cloates submarine canyons (indicated by the red square) in the eastern Indian Ocean off the coast of Western Australia. (b) Sampling sites within the Cape Range (top, $n=12$) and Cloates (bottom, $n=4$) submarine canyons, with ROV and CTD (red dots) and ROV only (blue) sites indicated. All sites within the Cape Range Canyon had a visual transect conducted.

TABLE 1 | Primer information for PCR assays used in this study, including marine eDNA metabarcoding from the Cape Range and Cloates canyons, as well as taxon-specific amplification of tissue-derived samples for reference database generation.

PCR assay	Target taxa	Primer name	Sequence (5'-3')	Target length (bp)	Annealing temp (°C)	References
16S Fish	Bony Fish (eDNA)	16SF/D (F) 16S2R-degenerate (R)	GACCCTATGGAGCTTTAGAC CGCTGTTATCCCTADRGTAACCT	178–228	54	Berry et al. (2017) Deagle et al. (2007)
COI Leray	Animalia (eDNA)	m1CO1intF (F)	GGWACWGGWTGAACWGTWTAAYCCYCC	304–313	46	Leray et al. (2013)
COI (Folmer)	Arthropoda, Mollusca, Polychaeta, Scleractinia	jgHCO2198 (R)	TAIACYTCIAAYCAYAAARGAYATTGG	~710	40	Geller et al. (2013)
		LCOI1490 (F)	GGTCAACAAATCATAAAGATATTGG			Folmer et al. (1994)
COI (Meyer)	Arthropoda, Mollusca, Polychaeta, Scleractinia	HCO2198 (R)	TAAACTTCAGGGTGACCAAAAATCA	~658	40–44	Meyer (2003)
		dgLCOI1490 (F)	GGTCAACAAATCATAAAGAYATYGG			
COI (Geller)	Cnidaria (non-Scleractinia), Hemichordata, Asteroidea	dgHCO2198 (R)	TAAACTTCAGGGTGACCAAAAAYCA	~658	48	Geller et al. (2013)
		jgLCOI1490 (F)	TITCIACIAAYCAYAAARGAYATTGG			
COI (Arndt)	Holothuroidea	jgHCO2198 (R)	TAIACYTCIGGRTGICCRARAAYCA	~692	50	Arndt et al. (1996)
		COlef (F)	ATAATGATAGGAGG[A/G]TTTGG			
COI (Ward)	Bony Fish	COler (R)	GCTCGTGT[A/G]TCTAC[A/G]TCCAT	~555	50	Ward et al. (2005)
		FishF1 (F)	TCAACCAACCACAAAGACATTGGCAC			
16S (Palumbi)	Bony Fish	FishR1 (R)	TAGACTTCTGGGTGGCCAAAAGAAATCA	~550	50	Palumbi (1991)
		16Sar-L (F)	CGCCTGTTTATCAAAAACAT			
		16Sbr-H (R)	CCGGTCTGAACTCAGATCACGT			

Note: The 16S Fish and COI Leray assays were used for eDNA metabarcoding targeting the 16S and COI gene regions respectively, while additional COI and 16S primers were used for tissue-based barcoding. In the primer name, the “(F)” refers to the forward primer and “(R)” to the reverse primer.

2.2 | Generation of Custom DNA Reference Sequences

Curated reference databases for select gene regions enhance the utility and taxonomic resolution of current metabarcoding databases (Nester et al. 2020; Hleap et al. 2021; Leray et al. 2022). Building on this approach, custom databases for cytochrome c oxidase subunit I (COI) and 16S ribosomal RNA (16S; fish only) gene regions were created using tissue subsamples collected on-board the RV *Falkor* from specimens identified to the lowest taxonomic level possible (Table S2). To create a COI database from specimens found at the same localities, tissue subsamples from 203 Western Australian Museum (WAM) voucher specimens were extracted using a DNeasy Blood and Tissue Kit (Qiagen) and amplified using taxon-specific COI primers (see Table 1 for primer details), and sequencing was performed bidirectionally at the Australian Genome Research Facility (AGRF, Perth). For the 16S fish database, tissue from 32 voucher specimens was removed from 100% ethanol, dried, and subsampled to approximately 20 mg. DNA was extracted using a QIAcube extraction platform (Qiagen) with the DNeasy Blood and Tissue Kit, quantified with a Qubit Fluorometer (Thermo Fisher Scientific) and amplified using fish-specific 16S primers (see Table 1), with sequencing performed bidirectionally at Macrogen (Seoul, South Korea). Reference sequences were assembled for each sample using Geneious v.10.1.3. All sequences (COI and 16S) were deposited into GenBank (accession numbers available in Table S3).

2.3 | eDNA Field Sampling

Water samples ($n = 178$) were collected from 16 sites within the Cape Range and Cloates canyons (Figure 1). Ten-liter water samples were collected in Niskin bottles using a Conductivity, Temperature, Depth (CTD) rosette and the ROV *SuBastian*. In total, 148 samples were collected using the CTD and 30 samples using the ROV. All ROV samples were taken on the seafloor (1745–4510 m), with two 5 L samples taken at the beginning and end of the 500 m transect conducted at each site. These samples were subsequently treated as technical replicates and merged in statistical analyses. The 5 L samples were combined to make one 10 L sample for the start and end of each transect. The volume was selected based on the CTD's Niskin bottle capacity, and the ROV *SuBastian's* configuration of four 5-L Niskin bottles. CTD samples were taken at depths of surface (0–10 m), 200, 500, 1000 m, and seafloor (2020–4370 m), with 3 replicate 10 L water samples taken at each depth, which were subsequently treated as technical replicates and merged in statistical analyses. Water samples were individually filtered within 3 h of collection using a Sentino microbiology peristaltic pump (Pall Life Sciences) through 47 mm diameter, 0.45 μ m pore size polyethersulfone filter membranes (Pall Life Sciences). Pore size was selected as samples were anticipated to have low turbidity and subsequently high filtering rates. Filter papers were cut in half and immediately preserved at -80°C , with one half to be analyzed and one to be stored as a reserve and a form of eDNA biobanking. Filtration equipment was soaked in a 10% bleach solution (changed daily) for a minimum of 15 min and rinsed with desalinated and filtered water in between each sample. One-liter samples of the bleach solution used for decontaminating filtration equipment, along with desalinated water used to rinse residual bleach, were

taken at the end of each filtering day and processed as filtration controls to confirm that no amplifiable DNA persisted following decontamination. Filter membranes were transported to a quarantine facility within the Trace & Environmental DNA (TrEnD) Laboratory (Perth, Western Australia) for further processing.

2.4 | DNA Extraction and Metabarcoding

DNA was extracted from half of the filter membrane using a DNeasy Blood and Tissue Kit (Qiagen) with the following modifications: 540 μ L of ATL lysis buffer, 60 μ L of Proteinase K, and a 12-h digestion at 56°C . Extraction controls (i.e., no sample) were processed in parallel with all samples to detect any laboratory or between-sample contamination. Extraction was completed using a QIAcube (Qiagen) DNA extraction system. Final DNA extracts were eluted in 100 μ L of AE buffer and stored at -20°C .

DNA was amplified using the 16S Fish assay and the COI Leray assays (Table 1). The COI Leray assay broadly targets all Animalia taxa and was selected to maximize diversity detections. Comparatively, the 16S Fish assay has been optimized to specifically target bony fish. Negative controls were included on each qPCR plate to control for contamination. To reduce the likelihood of index-tag switching and chimera production, metabarcoding was performed using fusion-tagged primers consisting of an Illumina-compatible adapter sequence, a unique multiple identifier (MID) tag, and a respective primer sequence for each assay. Each sample and control were processed in duplicate using the same MID tag to reduce stochasticity for species with low amounts of template DNA. qPCR reactions (25 μ L) consisted of the following: 0.5 μ M forward and reverse primer (Table 1), 2 mM MgCl_2 , 1 $\times$ AmpliTaq Gold PCR buffer, 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.25 mM dNTPs (Astral Scientific), 0.1 mg BSA (Fisher Biotech), 0.6 μ L of 5 $\times$ SYBR Green dye (Life Technologies), and 4 μ L template DNA, made to volume with Ultrapure Distilled Water (Life Technologies). qPCR amplifications were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems) under a three-step cycling regime. Thermocycler conditions were as follows: 95°C for 5 min, followed by 50 cycles of 95°C for 30 s, annealing temperature (Table 1) for 30 s, and 72°C for 45 s, completed by a 72°C elongation step for 10 min. All qPCR reactions were prepared in dedicated clean room facilities at the TrEnD Laboratory (Curtin University). Resulting amplicons were pooled at equimolar ratios, size selected using a Pippin Prep (Sage Science) and purified using a QIAquick PCR Purification Kit (Qiagen). The final libraries were quantified using a QIAxcel Advanced System (Qiagen) and a Qubit Fluorometer (Thermo Fisher). Libraries were sequenced on an Illumina MiSeq platform using either a 500-cycle MiSeq V2 Reagent Kit for paired-end sequencing (COI Leray) or 300-cycle MiSeq V2 Reagent Kit for single-end sequencing (16S Fish).

2.5 | Bioinformatics and Taxonomic Assignments

Bioinformatics and taxonomic assignments of sequencing data were performed using eDNAFlow, a fully automated workflow that processes eDNA data from raw sequences to curated and noncurated zero-radius operational taxonomic units

(ZOTUs) and their abundance tables (Mousavi-Derazmahalleh et al. 2021). In brief, sequences were quality filtered, demultiplexed, denoised, and erroneous sequences removed using a combination of AdapterRemoval (Schubert et al. 2016), OBITools (Boyer et al. 2016), and Usearch (Edgar 2010) via Setonix, a HPE Cray EX supercomputer at the Pawsey Supercomputing Centre (Kensington, Western Australia). Default eDNAFlow pipeline parameters were used for sequence filtering and denoising, with the exception that no primer mismatches were permitted during primer trimming. ZOTUs were retained only if supported by at least four reads (minsize=4), and all retained ZOTUs exceeded 100bp in length. Resulting ZOTUs were queried against NCBI's GenBank nucleotide database (accessed June 2025) and two custom reference databases of WAM vouchered specimens obtained during the voyage (herein WAM custom database) using BLASTn. For taxonomic assignment, BLASTn results were filtered using thresholds of $\geq 90\%$ sequence identity and 100% query coverage, retaining up to the top 10 target sequences per query. ZOTUs were assigned taxonomy using a lowest common ancestor python script within eDNAFlow (Mousavi-Derazmahalleh et al. 2021). Taxonomic assignments were then manually curated against knowledge of species distributions using the World Register of Marine Species (WoRMS Editorial Board 2022), Atlas of Living Australia (ALA—ala.org.au), Fishes of Australia (fishesofaustralia.net.au), FishBase (Froese and Pauly 2025), and the WAM collections database. Final curated assignments, including putative new records and range extensions, were reviewed by taxonomic experts at the Western Australian Museum to assess the plausibility of identifications and the validity of interpreting detections as range extensions based on existing distributional knowledge (Tables S4 and S5). To present putative new occurrence records or range extensions we required taxa to be either (i) a species in a monotypic genus (with $< 96\%$ identity to closest relative), (ii) have all congeneric taxa (currently described as of June 2025) barcoded for the relevant gene region of assay, and/or (iii) have no prior occurrence recorded on the WoRMS or ALA.

ZOTUs assigned to contaminants (i.e., present in field controls), non-metazoan, and non-marine metazoan were removed and excluded from further analysis. These detections were primarily humans (*Homo sapiens*), Bacteria, and Chromista. Several species of fish (*Arripis georgianus*, *Katsuwonus pelamis*, *Thunnus alalunga*, *T. albacares* and *Mugil cephalus*) were identified as potential contaminants due to their use as bait in fish and crustacean traps throughout the voyage. To be conservative, ZOTUs assigned to these species were removed regardless of if the species were found in the region.

2.6 | Statistical Analysis

Taxonomic datasets for Animalia (COI Leray) and fish (16S Fish) were analyzed separately to account for potential assay biases. Overall differences in richness and diversity between canyons (Cape Range and Cloates) and depths (CTD samples only: surface, 200, 500, 1000m, and bottom) were explored visually in R Studio (v.4.2.1; R Studio Team 2022). To examine the taxonomic resolution of each assay (i.e., the ability to accurately distinguish taxa), the proportion of assignments matched to various

taxonomic levels ("unclassified", order, family, genus, and species) was calculated. A family-level phylogenetic tree was reconstructed with *phyloT*, using families filtered at $> 94\%$ sequence identity and matched to their corresponding NCBI taxonomic identifiers. The resulting Newick tree was imported into R and visualized as a circular cladogram with *ggtree* (Yu et al. 2017).

Differences in species richness between canyons and depths were assessed using ANOVAs. Triplicate samples derived from the same Niskin cast were treated as technical replicates (pseudo-replicates) and aggregated prior to all statistical analyses. Differences in community composition were explored using PRIMERv7 with the PERMANOVA + add-on (Anderson et al. 2008). Data were transformed into presence-absence matrices and converted to a Jaccard matrix. A permutational multivariate analysis of variance (PERMANOVA) was used to test community variation among canyons and depths (CTD samples only), with canyon and depth treated as fixed factors and site included as a random factor nested within canyon to account for repeated sampling across depths within sites. PERMANOVA was run using Type III (partial) sums of squares with permutation of residuals under a reduced model (9999 permutations), with permutations constrained due to the nested site design. Pairwise comparisons were conducted for significant main effects and interactions using the same model structure to identify where community composition differed. Community composition was visualized by principal coordinate analysis (PCO). Similarity percentage analyses (SIMPER) were also conducted in PRIMER v.7 to identify taxa contributing most to pairwise dissimilarity between canyons and depths. Lastly, to examine potential depth stratification, each assay was analyzed separately across the sampling depths.

Comparisons between eDNA metabarcoding and ROV transects could only be made at the phylum level due to the low taxonomic resolution of the ROV annotations. eDNA samples were subset to include only those taken at sites where ROV transects were completed and the proportion of phyla detected at each transect was then compared between the methodologies.

All data generated and R scripts created have been deposited in a Zenodo repository (<https://doi.org/10.5281/zenodo.18581057>). This includes sampling information, raw sequencing data, morphology data, ZOTU tables for both metabarcoding assays, and results of statistical analyses.

3 | Results

3.1 | eDNA Sequencing Statistics

A total of 11,396,634 sequencing reads were obtained using the COI Leray and 16S Fish metabarcoding assays. For the COI Leray assay 2807 ZOTUs were assigned, of which 1051 (37.44%) were retained post-curation and contamination removal. The relatively high proportion of discarded COI ZOTUs primarily reflected non-target amplification which is a well-recognized limitation of universal COI primers in environmental samples (Collins et al. 2019; Hintikka et al. 2022), and stringent post-denoising taxonomic filtering. Taxonomic assignments using

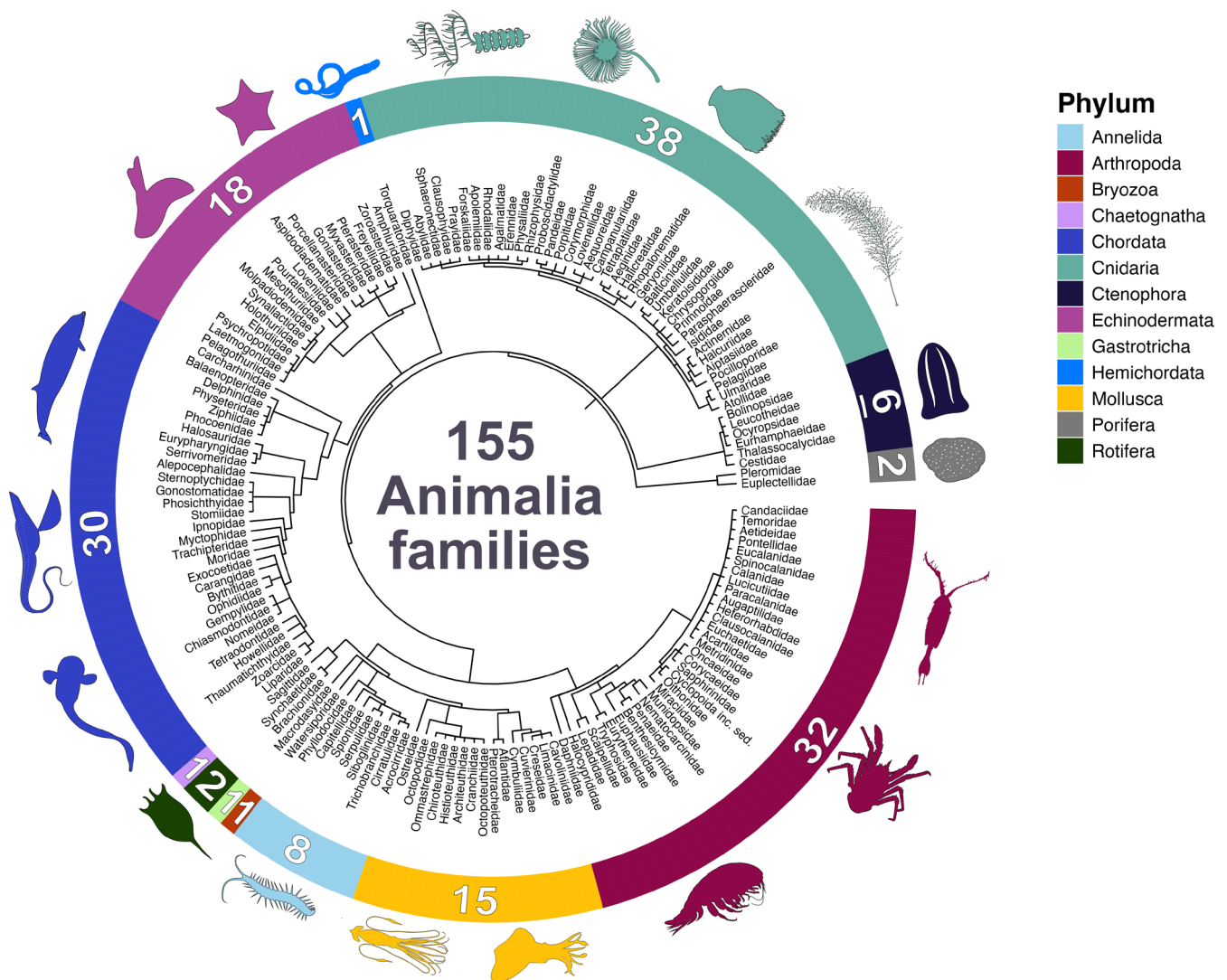


FIGURE 2 | Family level taxonomic phylogram of Animalia taxa detected in the Cape Range and Cloates canyons in the Indian Ocean. eDNA metabarcoding was performed using the COI Leray assay. Taxa silhouettes are colored according to the phyla they represent.

the NCBI reference database were further refined with the WAM custom database, enabling identification of 53 previously unassigned ZOTUs corresponding to 41 unique taxa (77.4% resolved to species level). For the 16S Fish assay, 592 ZOTUs were assigned, of which 544 (91.89%) were retained post-curation and contamination removal. The use of the WAM custom database resolved an additional 26 fish taxa (class: Actinopterygii and Chondrichthyes) from 67 ZOTUs, with 13 taxa (46.43%) resolved to species level.

3.2 | eDNA Diversity and Richness

The two metabarcoding assays collectively identified 234 species, representing 125 families in 59 orders, providing new baselines for 11 different phyla in the biologically unexplored Cape Range and Cloates canyons. A total of 366 Animalia taxa (1047 ZOTUs) were detected using the COI Leray assay, with the largest proportion resolved to species level (179), and the remainder resolved to genus (97), family (48), order (24),

class (16), and phylum (2). Predominant phyla recovered were Cnidaria (38 families), Arthropoda (32 families), Chordata (30 families), Echinodermata (18 families), and Mollusca (15 families; Figure 2). This trend was reflected in the distribution of assigned reads, with siphonophores (Cnidaria: Siphonophorae; 41.7%) and calanoid copepods (Arthropoda: Calanoida; 37.1%) together comprising nearly 79% of the total reads. Across the 178 samples analyzed (148 CTD and 30 ROV), the most frequently occurring species were siphonophore species *Lensia campanella* (74 detections), *Apolemia* sp. (42 detections), and *Exocoetus volitans* (42 detections).

Using the 16S Fish assay, 154 fish taxa (544 ZOTUs) were detected (class: Actinopterygii and Chondrichthyes), representing 61 fish families (Figure 3). Of these taxa, the largest proportion were assigned at a species level (72), followed by genus (49), family (22), order (9), class (1), and phylum (1). Predominant fish families included lanternfish (Myctophidae; 9 genera), cusk-eels (Ophidiidae; 7 genera), and snake mackerels (Gempylidae; 6 genera). Read distribution was dominated by Ophidiidae (28.6%),

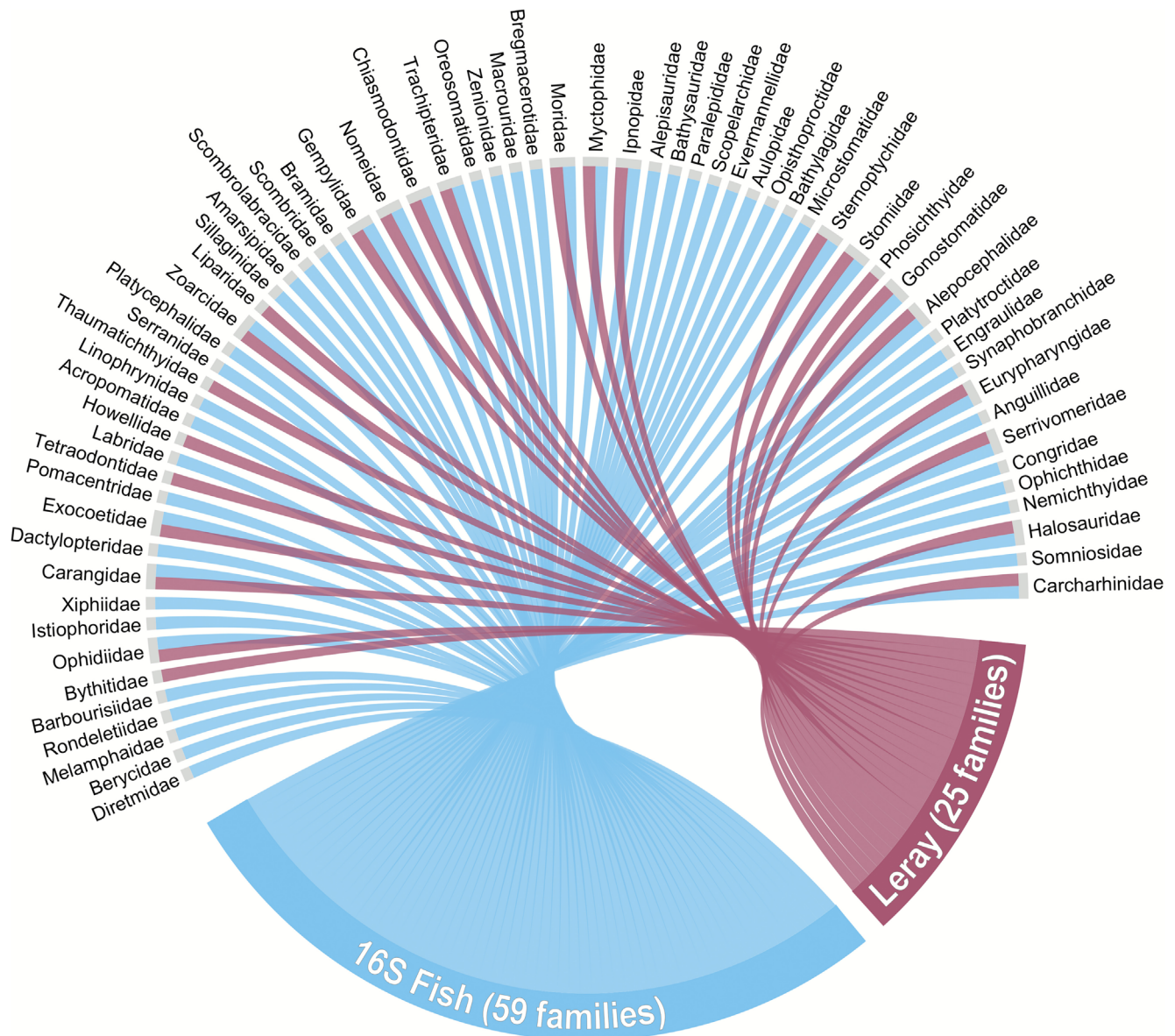


FIGURE 3 | Family level taxonomic phylogram of bony fish (Class: Actinopterygii) and shark (class: Chondrichthyes) taxa detected via eDNA in the Cape Range and Cloates submarine canyons in the Indian Ocean. eDNA metabarcoding was performed using the 16S Fish (blue) and COI Leray (red) assays. The two metabarcoding assays yielded 65 fish and shark families in total.

Exocoetidae (17.3%), Myctophidae (14.5%), and Zoarcidae (13.5%), which together accounted for 73.9% of the total reads. Across the 178 samples analyzed (148 CTD and 30 ROV), the most frequently detected fish species were *Seleniolytus* sp. and *Barathrites iris*, with 73 and 72 respective detections. A total of 40 fish families were exclusively detected using the 16S Fish assay, while a distinct subset of six families were detected only using the COI Leray assay (Figure 3).

A total of 83 taxa were identified as new records (genus or species), or range extensions (genus or species) across both eDNA assays (see Section 2.5; Table S6), and a subset is presented in Figure 4 and Table 2. Of these taxa, 27.66% were putative new occurrence records or range extensions that blasted against the WAM custom database. The remaining 72.34% were based on eDNA hits to the NCBI database.

3.3 | eDNA-Derived Patterns Across Canyons

The number of unique species detections was higher in Cape Range Canyon (12 sites) than Cloates Canyon (4 sites) for both the COI Leray (Figure 5a) and 16S Fish assay (Figure 5d). However, when averaged by site, the COI Leray assay indicated higher richness in Cloates Canyon, averaging 7.5 species per site (± 2.01) compared with 4.29 (± 0.71) in Cape Range. For the 16S Fish assay, averages were similar between canyons, with 4.44 (± 0.21) species per site in Cape Range and 4.37 (± 0.25) in Cloates. Despite these differences in overall and mean richness, no significant differences in species richness between canyons were detected for either assay (COI Leray: $F_{1,39} = 3.148$, $p = 0.084$; 16S Fish: $F_{1,39} = 0.003$, $p = 0.96$). Community composition differed significantly between the canyons for both the COI Leray (Pseudo- $F = 1.623$, $p = 0.002$) and 16S Fish assay

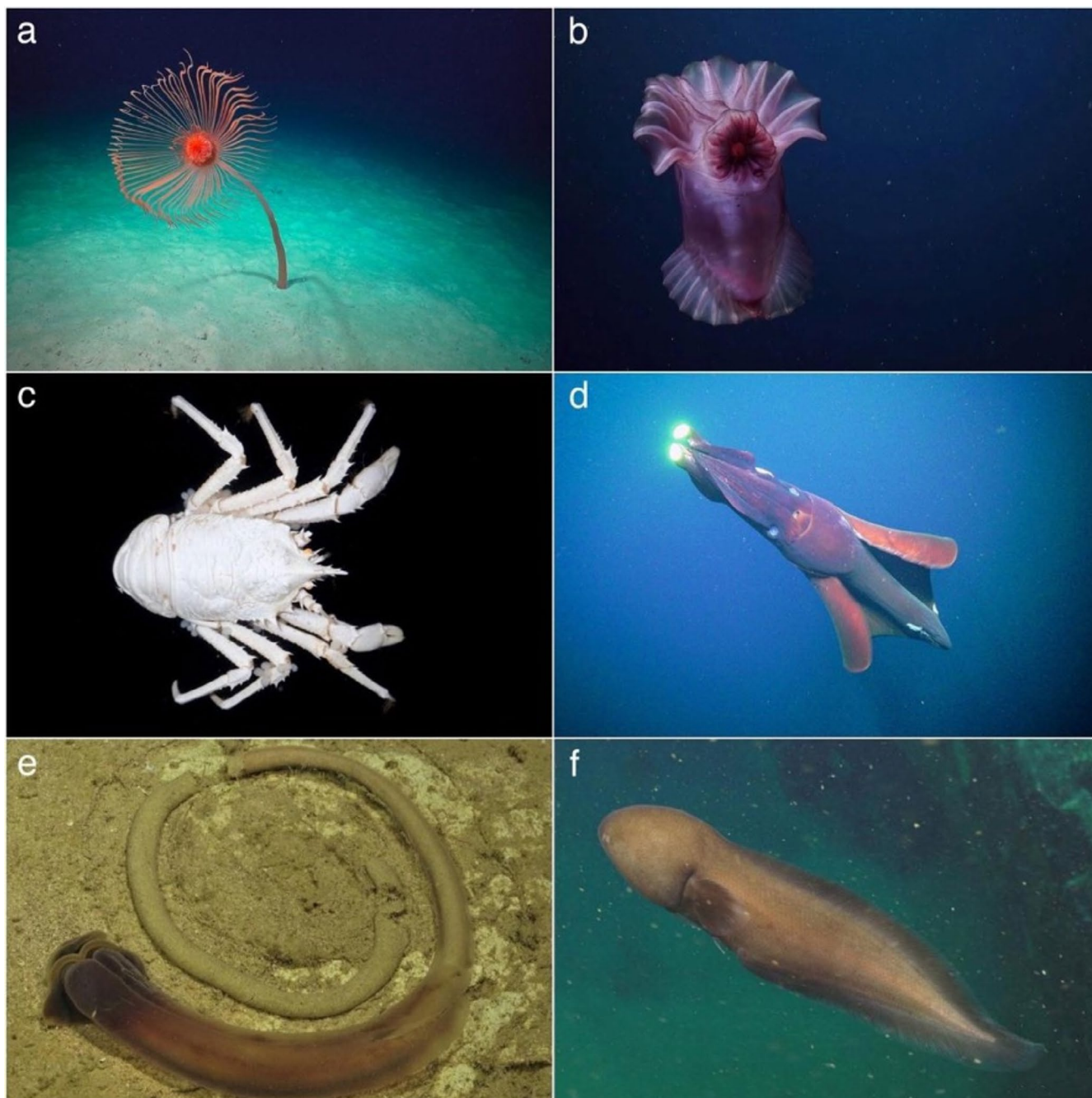


FIGURE 4 | A subset of taxa of interest detected in the Cape Range and Cloates submarine canyons in the Indian Ocean (Western Australia). Taxa were detected using eDNA metabarcoding with the COI Leray and 16S Fish assays. Taxa include (a) giant hydroid *Branchiocerianthus* sp.; (b) deep-sea cucumber *Enypniastes* sp.; (c) squat lobster *Munidopsis* cf. *subsquamosa*; (d) Dana octopus squid *Taningia* sp.; (e) acorn worm *Tergivelum* sp.; and (f) faceless cusk eel *Typhlonus nasus*.

(Pseudo- $F=3.882$, $p=0.0005$). However, strong separations between canyon community composition were only visually evident in the fish (16S Fish) PCO (Figure 5f). No separations between canyon community composition were visually evident in the COI Leray PCO (Figure 5c), indicating that differences between depths were greater than differences between canyons for broader Animalia taxa (Figure 5b).

SIMPER analysis identified the taxa contributing most to the dissimilarity between Cape Range and Cloates canyons using the COI Leray assay (Table S7). Communities in both canyons were

dominated by siphonophores (Family: *Sphaeronectidae*, Genus: *Sphaeronectes*) and hydrozoans (Class: Hydrozoa), which drove much of the within-group similarity. Despite these shared dominant taxa, the canyons were highly distinct overall (average dissimilarity = 82.8%). Cape Range was characterized by higher detections of the siphonophores *Apoemia* and *Rosacea*, whereas Cloates was distinguished by the lobate ctenophore *Leucothea* and benthic fishes (*Pachycara* sp., *Barathrites iris*). For the 16S Fish assay (Table S8), Cape Range communities were characterized by detections of flyingfishes (Genus: *Exocoetus*), lanternfishes (Genus: *Diaphus*, Family: Myctophidae), and the eelpout

TABLE 2 | A subset of taxa of interest detected in the Cape Range and Cloates submarine canyons (Indian Ocean–Western Australia) using eDNA.

Phyla	Species	Common name	Database	Distribution	Specimen collected	Comment
Annelida	<i>Swima</i> sp.	—	WAM	NE & W Pacific, Celebes Sea (Philippines)	Y	New record for Australia. Unresolved taxon, possible new species.
	<i>Teuthidodrilus</i> sp.*	Squidworm	WAM	Celebes Sea (Philippines)	Y	New record for Australia. Unresolved taxon, possible new species.
Arthropoda	<i>Eurythenes maldoror</i>	—	WAM	Weddell Sea, Argentinian Basin, N. Atlantic & Pacific	Y	New Indian Ocean record.
	<i>Munidopsis</i> cf. <i>subsquamosa</i>	Squat lobster	WAM	NE Pacific; NW & SW Atlantic	Y	New Indian Ocean record.
	<i>Nigmatullinus acanthitelsonis</i>	—	WAM	Atlantic	Y	New record for Australia.
	<i>Acanthonus armatus</i>	Bony-eared assfish	WAM	Circumtropical	Y	Detected recently in Gascoyne MP by Bessey et al. (2025); our record corroborates regional presence.
Chordata	<i>Alcockia</i> sp.*	Cusk-eel	WAM	W. Atlantic & E. Pacific	Y	New record for Australia. Unresolved taxon, possible new species.
	<i>Amarsipus carlsbergi</i> *	Bagless glassfish	NCBI	Indo-Pacific	N	New East Indian Ocean record.
	<i>Apagesoma australe</i>	—	NCBI	Crozet Islands (Southern Ocean)	N	New species and genus record for Australia.
	<i>Barathrites iris</i>	Cusk-eel	WAM	North-east Atlantic, North Pacific	Y	New species and genus record for Australia.
	<i>Bathysaurus ferox</i>	Deepsea lizardfish	NCBI	Circumglobal	N	Northerly range extension. Consistent with Bessey et al. (2025) detections in Gascoyne MP.
	<i>Coryphaenoides armatus</i>	Abyssal grenadier	NCBI	Circumglobal	N	New record for WA.
	<i>Evermannella</i> sp.	Sabretooth	NCBI	Indo-Pacific	N	New species and genus record for WA.

(Continues)

TABLE 2 | (Continued)

Phyla	Species	Common name	Database	Distribution	Specimen collected	Comment
Eurypharynx pelecyanoides Halosauropsis macrochir Inops agassizii Kogia breviceps Neophocaena phocaenoides Pachycara sp. Paraliparis sp. Penopus japonicus Pseudorca crassidens Rhadinesthes decimus* Seleniolytus sp. Somniosus sp. Stemonidium hypomelas* Typhlonus nasus Zenion japonicum Ziphius cavirostris		Pelican eel	NCBI	Circumglobal	N	Southerly range extension
		Abyssal halosaur	NCBI	Circumglobal	N	Northerly range extension.
		Grideye spiderfish	WAM	Indo-Pacific	Y	New record for WA.
		Pygmy sperm whale	NCBI	Circumglobal	N	Possible range extension; elusive species.
		Indo-Pacific finless porpoise	NCBI	South Pacific	N	New species and genus record for Australia.
		Eelpout	WAM	Circumglobal	Y	New record for Australia. Unresolved taxon, possible new species.
		Snailfish	NCBI	Circumglobal	N	New genus record for mid-WA
		Cusk-eel	WAM	West Pacific	Y	New record for Australia.
		False killer whale	NCBI	Circumtropical	N	Evidence of genetically independent resident coastal population
		Slender snaggletooth	NCBI	Circumglobal	N	New species and genus record for Australia.
		Eelpout	WAM	Southern Ocean (Antarctic)	Y	New species and genus record for WA. Unresolved taxon, possible new species.
		Sleeper shark	NCBI	Circumpolar and temperate oceans	N	New species and genus record for WA; range extension for Somniosus.
		Black serrivomerid eel	NCBI	Circumglobal	N	New record for WA.
		Faceless cusk eel	WAM	Indo-Pacific	Y	New record for WA.
		Japanese dory	NCBI	W. Pacific	N	New species and genus record for WA; large range extension.
		Cuvier's beaked whale	NCBI	Circumglobal	N	Northerly range extension.

(Continues)

TABLE 2 | (Continued)

Phyla	Species	Common name	Database	Distribution	Specimen collected	Comment
Cnidaria	<i>Apolemia lanosa</i>	—	NCBI	Eastern Pacific	N	New species and genus record for WA.
	<i>Athorybia rosacea</i>	—	NCBI	Atlantic, Mediterranean, Pacific	N	New East Indian Ocean record.
	<i>Branchiocerianthus</i> sp.	Giant hydroid	WAM	Circumglobal	Y	First giant hydroid in Australia.
	<i>Erenna</i> sp.	—	NCBI	Atlantic & Pacific	N	New species and genus record for WA.
	<i>Halistemma rubrum</i>	—	NCBI	Cosmopolitan	N	New record for WA.
	<i>Hydrichthys boycei</i>	—	NCBI	N. Atlantic & N. Pacific	N	New genus record for Australia & Indian Ocean.
	<i>Lilyopsis medusa</i>	—	NCBI	Atlantic	N	New species and genus record for WA.
	<i>Marrus</i> sp.	—	NCBI	Atlantic, Antarctic, Arctic, East Pacific	N	New East Indian Ocean record.
	<i>Pandea</i> sp.	—	NCBI	Circumglobal	N	New species and genus record for WA.
	<i>Rhizophysa eysenhardtii</i>	—	NCBI	Atlantic & Mediterranean	N	New record for Indian Ocean; unresolved taxon.
Ctenophora	<i>Eurhamphaea</i> sp.	—	NCBI	Tropical cosmopolitan	N	New species and genus record for WA.
	<i>Velamen parallelum</i> *	—	NCBI	Circum(sub)tropical	N	Unresolved taxon, possible novel diversity. Requires specimen confirmation.

(Continues)

TABLE 2 | (Continued)

Phyla	Species	Common name	Database	Distribution	Specimen collected	Comment
Echinodermata	<i>Echinostira phiale</i>	Deep-sea echinoid	NCBI	North Atlantic	N	New record for WA.
	<i>Eynyniastes</i> sp.*	Deep-sea cucumber	WAM	Circumglobal	Y	New record for north WA.
	<i>Evoplosoma claguei</i>	Deep-sea corallivore	NCBI	NW Atlantic & Pacific	N	New Indian Ocean record.
	<i>Freyella</i> sp.	Deep-sea starfish	NCBI	Circumglobal	N	New species and genus record for WA.
Hemichordata	<i>Heligmaster kanaloa</i>	—	WAM	North Pacific	Y	New record for Australia.
	<i>Sibogaster niesenii</i>		WAM	North Pacific	Y	New record for WA.
	<i>Tergivelum</i> sp.	Acorn worm	WAM	N. Atlantic & NW Pacific	Y	New record for Australia. Unresolved taxon, possible new species.
						New record or range extension.
Mollusca	<i>Architeuthis dux</i> *	Giant squid	NCBI	Circumglobal	N	New record or range extension.
	<i>Asperoteuthis acanthoderma</i>	Thorny whiplash squid	WAM	Central Indo-Pacific	Y	New East Indian Ocean record.
	<i>Helicocranchia pfefferi</i>	Banded piglet squid	NCBI	Tropical to subtropical Atlantic, Pacific, and Indian Oceans.	N	New record for WA.
Porifera	<i>Stigmatoteuthis hoylei</i>	Flowervase jewel squid	WAM	Tropical Indian & Pacific	Y	New record for WA.
	<i>Taningia</i> sp.	Dana octopus squid	WAM	Cosmopolitan	Y	Rare bioluminescent squid. Unresolved taxon, possible new species.
	<i>Teuthowenia megalops</i>	—	WAM	North Atlantic	Y	New Indian Ocean record.
	<i>Docosaccus</i>		NCBI	Antarctic waters, North Pacific, Bering Sea	N	New species and genus record for Australia.

Note: Taxa were detected using the COI Leray and 16S Fish metabarcoding assays, with assignments made using the custom Western Australian Museum (WAM) database or NCBI. Monotypic genera are indicated by an asterisk (*). Taxa are listed as "unresolved" where morphological and molecular assessments by the Western Australian Museum are ongoing, and species identities are yet to be confirmed (i.e., potential new or undescribed taxa).

COI Leray: Animalia taxa

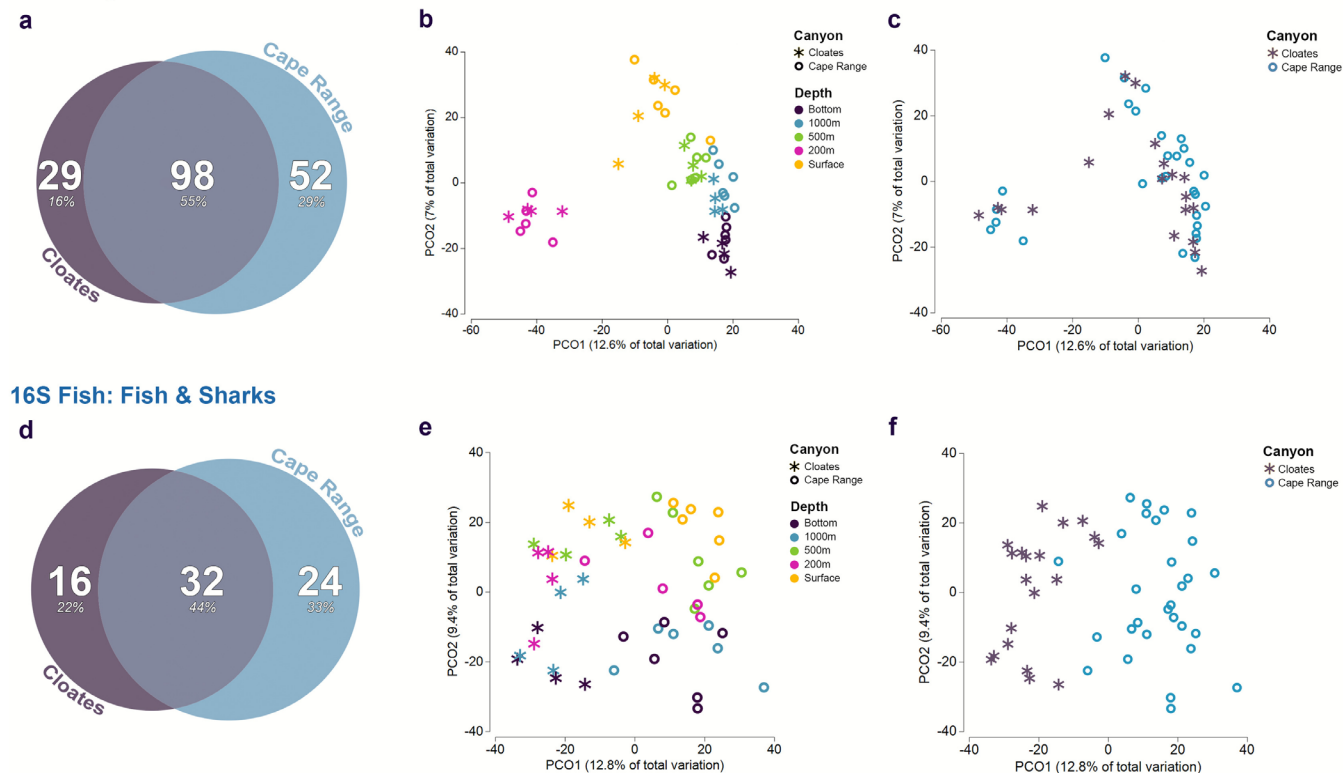


FIGURE 5 | (a) Venn diagram of unique and shared Animalia species detected using the COI Leray assay; (b–c) PCOs of Animalia taxonomic composition by depth (b) and by canyon (c); (d) Venn diagram of unique and shared fish and shark species detected using the 16S Fish assay; (e–f) PCOs of fish taxonomic composition by depth (e) and by canyon (f). Depths sampled were surface, 200, 500, 1000 m, and bottom in both the Cape Range and Cloates canyons.

Seleniolycus sp. (Family: Zoarcidae). In contrast, Cloates communities were dominated by cusk eel *Barathrites iris* (Family: Ophidiidae), with additional detections of *Pachycara* sp. (Family: Zoarcidae) and *Exocoetus*. Overall, Cape Range and Cloates remained highly distinct in composition (average dissimilarity = 80.0%), with the strongest contributors to between-canyon differences being *Barathrites iris*, *Pachycara* sp., and *Seleniolycus* sp.

3.4 | eDNA-Derived Patterns Across Depth Gradients

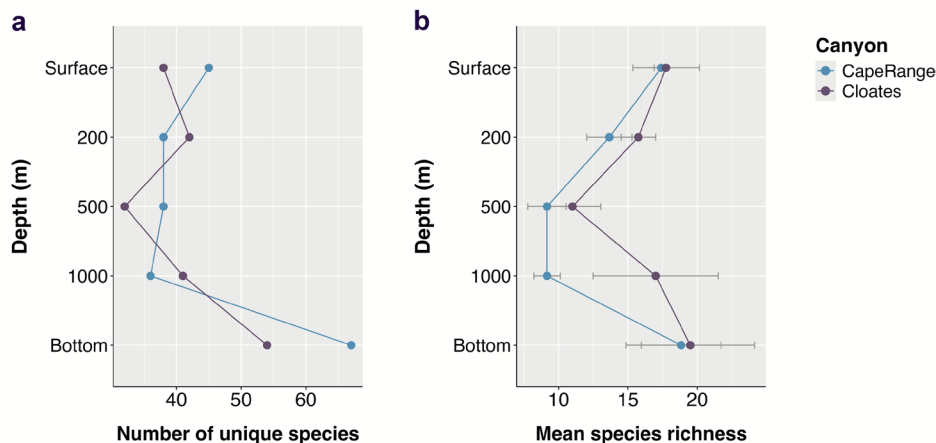
Both assays revealed depth-related differences in community composition, although the strength and taxonomic resolution of vertical structuring varied. Along the CTD depth gradient, the number of species detected per depth category was highest in the bathypelagic bottom water samples at both Cape Range (67) and Cloates (54) canyons using COI Leray (Figure 6a). Mean species richness was also highest in the bathypelagic bottom water, with comparable richness observed at the surface (Cape Range: 18.83 ± 2.88 and 17.4 ± 0.51 ; Cloates: 19.5 ± 4.62 and 17.75 ± 2.39 ; Figure 6b). These depth-related differences were significant ($F_{4,39} = 2.38$, $p = 0.002$), with richness higher in bottom and surface waters than at mid-depths (Figure 6b, Table S9). Comparatively, using the 16S Fish assay, species richness was highest in the epipelagic samples (200 m) at both Cape

Range (35) and Cloates canyons (26; Figure 6c). Mean species richness followed this trend, with 11.5 ± 1.61 and 9.75 ± 0.63 species at Cape Range and Cloates canyons respectively (Figure 6d), although no significant depth differences were detected ($F_{4,39} = 2.38$, $p = 0.068$).

Within the Animalia dataset (COI Leray), we found both surface and 200 m samples to be dominated by copepod taxa (Table S10). The 500 and 1000 m samples were dominated by Actinopterygii taxa, while bottom water samples were dominated by Actinopterygii and Hydrozoa. Within the fish (16S Fish) dataset, detections were broadly distributed across depths; although Ophidiiformes comprised the largest share of detections at all depths (Table S11).

Community composition significantly differed between CTD depths for both COI Leray (Pseudo- $F_4 = 4.2466$, $p < 0.0001$) and 16S Fish (Pseudo- $F_4 = 2.4$, $p < 0.0001$). Pairwise testing for COI Leray indicated that all depth categories were significantly distinct in composition. In contrast, pairwise comparisons for the 16S Fish assay revealed significant differences among most depth categories, although some adjacent depth comparisons were not significant (Tables S12–S15). For COI, samples clustered strongly by depth in PCO ordinations, with overlap increasing at greater depths (Figure 5b). SIMPER analysis revealed differences between depths were largely driven by higher detection rates of copepod taxa (*Clausocalanus paululus*, *C. furcatus*,

COI Leray: Animalia taxa



16S Fish: Fish & Sharks

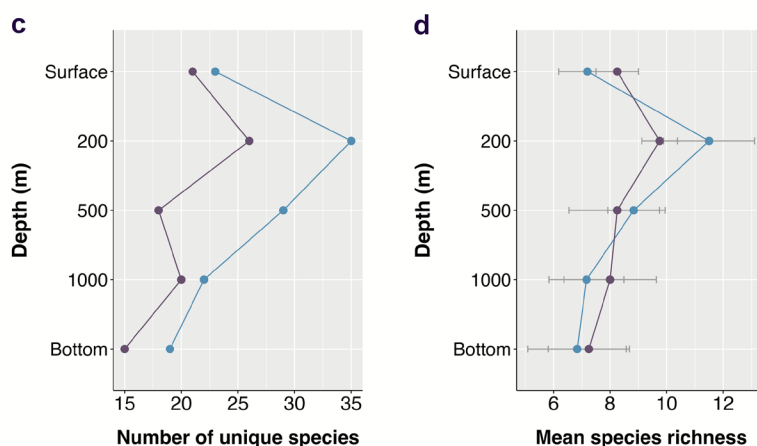


FIGURE 6 | Number of unique species and mean species richness across depths (surface, 200, 500, 1000 m, bottom) in the Cape Range and Cloates canyons using COI Leray (a–b) and 16S Fish (c–d) metabarcoding assays.

Farranula gibbula, *Calocalanus pavo*, and *Delius*) in the epipelagic zone (surface and 200 m), compared with siphonophores (*Apolesia*, *Rosacea*, and *Lensia campanella*) and hydrozoans (*Chuniphyes multidentata*) at deeper depths (500 m—bottom; Table S16). Read abundance patterns were consistent with these transitions, with copepods dominating surface samples (98.01% of reads), and siphonophores and hydrozoans dominating deeper waters (Table S17).

Although community composition significantly differed for 16S Fish samples, PCO analysis indicated that canyon identity was the dominant source of variation, with depth-related structuring being secondary (Figure 5e). Still, a broad separation was evident between shallow (surface–500 m) and deep (1000 m–bottom) assemblages (Figure 5e), with some clustering between surface and deep samples driven largely by Ophidiiformes (Figure S1). SIMPER results suggested that shallow assemblages were characterized by lanternfishes (*Diaphus*, *Myctophidae*) and flyingfishes (*Exocoetus*, *Cheilopogon*), whereas deeper assemblages were characterized by cusk eels (*Barathrites* sp., *Barathrites iris*), zoarcids (*Pachycara* sp., *Seleniolytus* sp.), and other deep demersal taxa such as *Bassozetus* and *Bathypterois* spp. (Table S18). Read abundance patterns supported this separation, with flying

fish (*Exocoetus*) showing the highest proportion of reads in surface samples (69%), while *Barathrites iris* was detected across all depths but contributed most strongly in bottom waters (27%) (Table S19).

3.5 | ROV Transect Comparison

Comparisons between eDNA (COI Leray) and ROV transects were done at the phyla level due to the low taxonomic resolution of ROV observations. Within the confinements of the transect, eDNA detected 12 phyla while ROV detected 9 phyla. No phyla detected were exclusive to ROV observations, while phyla exclusive to eDNA were Chaetognatha, Foraminifera, and Gastrotricha. These phyla were not recorded in ROV annotations, likely reflecting a combination of their small body size or the camera altitude (approximately 2 m above the seafloor), rather than true absence from the imagery. Composition of eDNA detections within the transect was primarily Cnidaria (20.2%), Chordata (19.3%), Arthropoda (13.2%), and Mollusca taxa (12.3%), while morphological ROV samples were dominated by Porifera (39.65%) and Echinodermata taxa (29.52%, Figure S2).

4 | Discussion

4.1 | Biodiversity of the Cape Range and Cloates Canyons

The biodiversity and community composition data obtained in this study provide new baselines for 11 different phyla in these biologically unexplored submarine canyons. Submarine canyons are widely recognized as biodiversity hotspots (De Leo et al. 2010; Robertson et al. 2020), their complex topography and enhanced productivity often result in distinct species assemblages and large aggregations of migratory species (Moors-Murphy 2014). This survey revealed the Cape Range and Cloates canyons support and provide habitat to a high diversity of taxa, with 234 species identified within 125 families using the COI Leray and 16S Fish assays.

Comparisons between Cape Range and Cloates canyons revealed notable differences in richness and community composition. Although compositional differences were supported by both the COI Leray and 16S Fish datasets, separation between canyon assemblages was more evident for fishes, suggesting that mobile taxa may respond more coherently to canyon-specific environmental conditions than the broader metazoan assemblage. Notably, each canyon exhibited unique taxonomic detections despite unequal sampling effort, indicating that the observed differentiation is unlikely to be attributable to sampling artifacts alone.

This compositional separation is consistent with the distinctive physical and ecological functioning of submarine canyons relative to adjacent slope habitats. Submarine canyons are inherently complex habitats characterized by distinctive hydrographic, sedimentological, and geochemical regimes that can vary markedly over short spatial scales (Ramírez-Llodra, Brandt, et al. 2010; Vetter et al. 2010; Fernandez-Arcaya et al. 2017). Rather than functioning as simple incisions into the continental slope, canyons modify shelf-slope exchange, particle transport, and near-bottom flow in ways that promote fine-scale heterogeneity in organic-matter supply, sediment dynamics, and habitat stability (Ramírez-Llodra, Brandt, et al. 2010; Robert et al. 2015; Fernandez-Arcaya et al. 2017). Such processes can facilitate species replacement among neighboring canyons without necessarily producing corresponding changes in overall richness, leading to elevated compositional turnover at relatively small along-slope distances (Vetter et al. 2010; Robert et al. 2015; McClain and Barry 2010).

Within the present study area, Cape Range and Cloates canyons are known to differ in hydrographic, sedimentological, and geochemical characteristics documented during the same survey programme (Post et al. 2022). It is therefore plausible that canyon-specific balances of organic-matter delivery, sediment mobility, and hard-substrate exposure contribute to the distinct assemblages observed here. Together, these findings suggest that adjacent submarine canyons may support biologically distinct communities shaped by local environmental forcing, rather than acting as interchangeable extensions of the surrounding slope. Future studies employing balanced sampling designs at finer spatial resolution, alongside explicit integration of hydrodynamic and biogeochemical context, will be essential

for resolving the mechanisms underpinning these patterns and assessing their persistence through time.

4.2 | Potential New Species and Records

Given the limited biological exploration of the Cape Range and Cloates submarine canyons, the potential for discovery and significant findings was high. Using eDNA metabarcoding, our study identified 83 taxa representing new records (genus or species) or range extensions (genus or species) across both assays, highlighting how little is known about these largely inaccessible deep-sea ecosystems. Several of the taxa we identified as potential first records for the Gascoyne region and adjacent northwestern Australian waters (e.g., *Acanthonus armatus*, *Bathysaurus ferox*, etc.) were also recently recovered by Bessey et al. (2025) in the Gascoyne Marine Park, highlighting rapid convergence among independent eDNA surveys in the north-west WA deep sea.

Most eDNA detections of poorly resolved taxa could be validated using tissue samples from specimens collected aboard the RV *Falkor*, like *Teuthidodrilus* sp. nov., a monotypic squidworm previously documented only in the Philippines (Osborn et al. 2011). However, several detections were based solely on eDNA matches to reference databases, without specimen verification. For example, ASVs that aligned best with *Velamen* aff. *parallelum* (96% similarity) had the next closest match to *Cestum veneris* (90% similarity). As these are the only two monotypic genera within the family, this suggests the presence of undescribed diversity within the family. Although such findings cannot confirm the existence of new species, they highlight gaps in current reference libraries and the potential for novel taxa to be present. As with visual surveys, expert taxonomic interpretation and subsequent specimen collection are essential to validate these findings. Nevertheless, the use of eDNA holds promise in guiding targeted survey efforts, not only for indicating potential new occurrence records or range extensions, but also for highlighting taxa that may represent unresolved or undescribed diversity.

Our study revealed a substantial number of species detections that could represent new records or range extensions for Western Australia (WA), the Indian Ocean, or Australia. These include the first record of giant hydroid *Branchiocerianthus* (specimen verified) in Australia, new WA record of the bony-eared assfish *Acanthonus armatus*, and new WA records of the elusive giant squid *Architeuthis dux*. The high abundance of potential new records and range extensions in deep-sea environments may be attributed to the homogeneity of their cold-water conditions, facilitating species' range expansion over large distances while maintaining environmental similarity. However, deep-sea environments are recognized as both highly diverse and heterogeneous (Levin et al. 2010; McClain and Barry 2010; Robertson et al. 2020), with studies demonstrating genetic differences among species within the same ecosystem over small and broad spatial scales (Quattrini et al. 2013; Schüller 2011; Watanabe et al. 2021; Xu et al. 2021). For instance, a recent investigation of the Southern Ocean identified 27 new species in the *Doris kerguelensis* species complex (Order: Nudibranchia) from a sample of 1000 specimens (Maroni et al. 2022). Therefore, the prevalence of putative new records and extensions is more likely

a reflection of the limited exploration of these environments and gaps in reference databases.

We also detected the presence of several migratory mammal species including the deep-diving Pygmy Sperm whale (*Kogia breviceps*) and Cuvier's Beaked Whale (*Ziphius cavirostris*). The occurrence of these species correlates with the abundance of prey organisms such as squid and fish, detected by eDNA methodologies. Both species are rarely encountered using traditional visual or acoustic monitoring methods due to their offshore distribution, cryptic behavior, and deep-diving ecology (Baird et al. 2006; Bloodworth and Odell 2008), highlighting the utility of eDNA as a valuable tool for gaining insights into the occurrence and ecology of elusive marine megafauna. Moreover, our study supports the notion of the Cape Range and Cloates canyons as biodiversity hotspots supporting large aggregations of predators and apex consumers (Sleeman et al. 2007). The detection of such diverse biota further emphasizes the ecological importance of preserving and safeguarding these unique and fragile deep-sea environments.

4.3 | eDNA Vertical Stratification

The choice of metabarcoding assay plays a significant role in influencing the observed vertical stratification patterns. Using the broadly amplifying COI Leray assay, we detected pronounced depth-associated structuring, with epipelagic taxa dominating surface and mid-water samples and bathypelagic/benthic taxa dominating deeper waters. By contrast, the 16S Fish assay resolved a weaker depth resolution, although a broad division was still apparent between shallow (surface—500 m) and deep (1000 m—bottom) assemblages. Canyon identity explained a substantial proportion of the variation in the fish dataset, yet depth-related structuring remained evident when contrasting epipelagic and bathypelagic communities.

These assay-dependent contrasts reflect both taxonomic coverage and the biological characteristics of the target groups. The COI Leray assay detects a wide range of Animalia taxa, including many benthic and sessile taxa, which likely contributed to the clearer vertical stratification patterns observed. Although many of these taxa possess pelagic larval stages that may occur at other depths (Baco et al. 2016; Gary et al. 2020; Hilário et al. 2015), eDNA metabarcoding does not resolve life stage, and signals therefore integrate contributions from both larval and adult sources. Consequently, depth-associated patterns are interpreted here at the level of broad taxonomic groups and assay targets rather than taxon-specific life-history strategies. Adult sedentary and sessile benthic organisms inhabiting bathyal or abyssopelagic depths typically exhibit limited vertical mobility (Baco et al. 2016; Hilário et al. 2015), a trait that may promote depth-confined eDNA signals more readily captured by universal assays such as COI Leray. In submarine canyon environments, additional contributions from organisms inhabiting steep walls and near-bottom habitats may also contribute to signals detected near the seafloor, reinforcing apparent depth-associated structuring. In contrast, the greater mobility and biomass of fish may lead to more extensive dispersal of DNA throughout the water column, weakening observed vertical patterns.

Life-history strategies and reproductive dynamics add further complexity to eDNA-derived vertical patterns. A well-recognized limitation of current eDNA metabarcoding approaches is the inability to distinguish different life stages of organisms, such as larvae or eggs. Although advances in species-specific assays and epigenetic approaches may eventually allow this distinction (Bylemans et al. 2016; Hayer et al. 2020; Holmes et al. 2022; Marshall and Stepien 2019; Ruiz et al. 2025; Takeuchi et al. 2019), most metabarcoding signals represent temporal and biological integration across multiple life stages. Biophysical modeling of deep-sea environments has demonstrated that vertical positioning strongly influences dispersal potential, with upward-swimming larvae able to disperse widely while those remaining near the seafloor tend to be locally retained (Gary et al. 2020). Similar patterns have been observed in Australia's southwest submarine canyons, where larvae of benthic invertebrates were largely retained within the canyon systems unless transported above the canyon rims, after which major currents facilitated long-distance dispersal (Kool et al. 2015).

The comparatively weak depth resolution observed for fishes in this study is therefore consistent with the combined effects of adult mobility, spawning behavior, and high biomass of fishes, rather than larval dispersal alone. Many deep-sea fishes, such as cusk-eels (family Ophidiidae), which were detected across all sampled depths using the 16S Fish assay, often reproduce near the sea floor, with fertilized eggs ascending into shallower waters and juveniles subsequently migrating back to typical adult depths (D'Onghia et al. 2015; Fernandez-Arcaya et al. 2013; Porcu et al. 2020; Nonaka et al. 2021; Takami 2022). This vertical coupling of life stages broadens the depth range over which fish-derived eDNA may be detected. Reproductive activity may further amplify this effect, as broadcast spawning and increased activity during reproductive periods have been shown to elevate eDNA concentrations across life stages (Collins et al. 2022), leading to more vertically diffuse signals in the water column. When combined with high adult mobility, these processes increase the likelihood that fish-derived eDNA is redistributed vertically, particularly during spawning periods, confounding vertical spatial patterns and obscuring depth boundaries. Diel vertical migration in some taxa may reinforce this effect by redistributing eDNA across depth strata, while persistence of DNA over hours to days can further reduce depth contrasts, even when behavioral shifts are pronounced (West et al. 2024; Trenkel et al. 2025).

Our findings therefore align with a growing body of work indicating that vertical stratification of eDNA can reflect organismal depth distributions and behavior, but typically at coarse resolutions (tens to hundreds of meters) and are strongly contingent on water-column structure and mixing regime (Jo et al. 2025). In relatively stable systems, eDNA can retain strong depth fidelity, as demonstrated by clear depth-specific patterns in pelagic fish eDNA in the Bay of Biscay, where detections were typically confined to the depth of release and deeper (Canals et al. 2021). In more complex or dynamically mixed environments, however, depth-related signals often persist but are partially attenuated, as reported by Bessey et al. (2025), who observed significant yet weaker separation of fish assemblages across continental shelf, slope, and abyssal habitats in the Indian Ocean.

Methodological choice further shapes how these biologically driven patterns are expressed in eDNA data. Fish-specific assays have been shown to outperform broadly amplifying metabarcoding approaches for resolving migration-linked depth structure in mobile taxa (Govindarajan et al. 2023; Trenkel et al. 2025), likely due to reduced taxonomic competition and improved sensitivity to fish-derived signals. However, even with appropriate markers, eDNA does not necessarily track instantaneous organism position. Mechanistic modeling indicates that vertical transport typically displaces eDNA by only tens of meters from its depth of origin (Allan et al. 2021), indicating that physical transport is limited. At the same time, because eDNA signals integrate shedding from multiple individuals over time, the effective vertical resolution at which community structure can be interpreted is often coarser, typically on the order of tens to hundreds of meters, and strongly contingent on water-column structure and mixing regime (Jo et al. 2025). As a result, pronounced behavioral changes do not always translate into sharp compositional turnover in eDNA. Consistent with this, West et al. (2024) observed little day-night change in fish eDNA community composition despite strong diel shifts in activity documented by diver surveys, highlighting how persistence of DNA over hours to days can blur both temporal and vertical contrasts.

In our study, the relatively weak but detectable depth-related structuring of fish eDNA likely reflects the interplay between biological factors and the highly dynamic physical setting of submarine canyons. Here, depth acts as an integrated proxy for multiple co-varying physical gradients, including temperature, pressure, and water mass structure, rather than a strict delineation of discrete hydrographic layers. Canyon systems concentrate diel vertical migrators (Genin 2004; Santora et al. 2018), displace deep-water species toward coastal zones (De Leo et al. 2010), and are subject to strong topographically induced upwelling (Moors-Murphy 2014; Sobarzo et al. 2001) and internal wave-driven mixing (Kunze et al. 2002; Waterhouse et al. 2017). Strong tidal currents near the shelf edge may further drive turbulence (Dipper 2022), redistributing DNA through the water column and weakening depth-specific signals. Together, these findings highlight that while vertical stratification of fish eDNA can be resolved, its expression depends on the interaction between life-history traits, assay choice, and local hydrodynamics, underscoring the need to consider physical oceanographic context when interpreting vertical eDNA patterns, particularly for mobile taxa in complex deep-sea environments.

4.4 | Comparisons Between eDNA Metabarcoding and ROV Transects

Comparisons between eDNA metabarcoding and ROV imagery identifications were conducted at the phylum level, taking into consideration the limited taxonomic resolution obtained from the ROV observations. eDNA metabarcoding was therefore a more effective approach for obtaining broad biodiversity data at a high taxonomic resolution, while ROV imagery facilitated environmental characterisations and an understanding of the fine-scale distribution of organisms. At the phylum level, eDNA metabarcoding detected 12 phyla compared to 9 phyla from ROV imagery, with no phyla unique to ROV observations.

The three phyla absent from ROV observations (Chaetognatha, Foraminifera, and Gastrotricha) were likely not recorded due to a combination of small body size, high mobility, and camera altitude (approximately 2 m above the seafloor), rather than true absence from the imagery.

The relative composition of detections also differed between methods at paired sampling locations. eDNA assemblages were dominated by cnidarians, chordates, arthropods, and mollusks, whereas ROV imagery was dominated by sponges and echinoderms. These contrasts likely reflect both organismal traits and methodological biases and sensitivities, with ROV imagery more effective at detecting large, sessile, and visually conspicuous taxa, while eDNA is better suited to capturing a broader range of phyla, including small, cryptic, or fragile taxa that are easily overlooked in visual surveys. Comparable patterns have been reported across both shallow and deep-sea systems, where molecular and visual approaches recover overlapping but largely complementary components of the community rather than equivalent biodiversity signals (Gold et al. 2023; Mirimin et al. 2021). In deep-sea environments in particular, shared detections between eDNA and video-based methods are often dominated by conspicuous or highly mobile taxa, while discrepancies reflect differences in detection probability linked to organismal biology, assay performance, and survey design (Fujiwara et al. 2022; Iguchi et al. 2024; Merten et al. 2021).

In addition, the broadly amplifying assays applied here are optimized for wide metazoan detection but may have reduced sensitivity for some deep-sea megafaunal groups (e.g., cephalopods, sponges and echinoderms), particularly where primer-template mismatches or incomplete reference databases persist. This pattern does not indicate a fundamental limitation of molecular approaches, but instead points to a need for the development of primers tailored to deep-sea taxa, which would greatly improve molecular recovery of these groups and facilitate more direct integration between eDNA and imagery-based approaches. Consequently, taxa that dominate benthic imagery may contribute proportionally less to the more taxonomically diverse eDNA assemblage.

An additional advantage of eDNA metabarcoding is its capacity for retrospective biological re-interrogation, rather than statistical reanalysis. Samples that are stored appropriately (biobanking; Jarman et al. 2018) can be revisited as new assays are developed, either to target taxonomic groups that were not the focus of the original study or to address new research questions. Likewise, existing metabarcoding datasets can be reanalyzed as reference databases improve and additional taxa are incorporated, increasing both the resolution and accuracy of taxonomic assignments over time. While visual datasets such as ROV imagery can be re-annotated, they remain constrained by the original imagery resolution, field of view, and taxonomic visibility. In contrast, eDNA samples retain molecular information that can be interrogated in fundamentally new ways as analytical tools advance, enabling retrospective analyses that extend well beyond the scope of the original study (Zhang et al. 2025). This flexibility is particularly valuable in remote or logistically challenging ecosystems like submarine canyons, where baseline

reference data are often scarce. In these settings, eDNA provides a means of building cumulative datasets that support ongoing biodiversity assessments and conservation initiatives.

4.5 | Methodological Advancements and Future Directions

The incomplete nature of reference databases poses a significant bottleneck in the taxonomic assignment of eDNA sequences. Consistent with prior research (Dugal et al. 2022; West et al. 2022), our results demonstrate that the development of locally curated or custom databases can greatly enhance taxonomic resolution and the proportion of assigned sequences. The use of a custom database of DNA reference sequences from specimens obtained on the voyage increased taxonomic assignment and resolution by 15%, underscoring the effectiveness of curated databases, particularly in hard-to-reach and unexplored environments. Importantly, these sequences have been deposited into a publicly accessible repository (GenBank), ensuring they can be drawn upon by future studies. Open access to high-quality, voucher-verified sequences provides a permanent resource that strengthens the accuracy of eDNA biodiversity surveys and supports the wider genomic research community.

Our study also supports previous findings (West, Richards, et al. 2020; Jeunen et al. 2022; Valsecchi et al. 2022) in highlighting the potential of eDNA metabarcoding to elucidate putative new species records, occurrences, and range extensions. While not all researchers will have the resources to confirm putative detections with taxonomic experts, meticulous post-assignment checks and quality control can greatly improve confidence (e.g., see West, Richards, et al. 2020). In our case, manual curation involved cross-checking automated assignments against knowledge of species distributions, with detections only considered putative new occurrences if they belonged to a monotypic genus or had complete congeneric coverage for the marker, and had no prior occurrence recorded on WoRMS or ALA. These curated assignments were then reviewed by taxonomic experts, ensuring that putative new records were supported by both sequence data and distributional plausibility. This layered quality control provides a rigorous framework for ensuring that eDNA-derived biodiversity records are both accurate and defensible.

Our research revealed significant differences in vertical stratification between the two assays employed, emphasizing the limitations of making assumptions or inferences about eDNA behavior based on a single taxonomic group or marker. This highlights the need for cautious assay consideration and thorough validation of eDNA metabarcoding protocols across diverse environments and taxa. We also acknowledge that our results may have been influenced by sampling time, which could have increased the presence of diel vertical migrators in our data. As our study aimed to rapidly characterize biodiversity in the target regions using multiple approaches, our sampling was necessarily opportunistic in nature. Nevertheless, future deep-sea eDNA studies would benefit from targeted sampling during specific times of day or night, or both, to account for diurnal

migrators. By doing so, a more comprehensive understanding of temporal dynamics and ecological patterns associated with eDNA in deep-sea environments can be achieved, while mitigating potential confounding factors and enhancing the robustness of the findings.

5 | Conclusion

This study presents the first comprehensive biological survey of the Cape Range and Cloates canyons (East Indian Ocean, Western Australia, FK200308) using eDNA metabarcoding (COI Leray and 16S Fish), ROV underwater imagery, and specimen collection. We characterized biodiversity spanning 11 different phyla and identified 83 taxa as putative new records, range extensions, or taxonomically unresolved taxa with the potential to represent undescribed diversity. These findings underscore the substantial knowledge gaps that persist in deep-sea ecosystems and highlight the value of eDNA for detecting elusive taxa, establishing novel records, and guiding targeted specimen collection. We also found that metabarcoding assay choice strongly influenced vertical stratification patterns, reflecting both the biological characteristics of the target taxa of each assay and canyon-specific environmental dynamics. Therefore, understanding the intricate relationship between eDNA-derived biodiversity patterns and the underlying biological, ecological, and physical factors is imperative for accurately interpreting these patterns. By demonstrating the efficacy of eDNA metabarcoding in submarine canyons, our study points to its broader potential in exploring uncharted deep-sea ecosystems, guiding targeted surveys, and advancing conservation strategies as anthropogenic pressures intensify.

Author Contributions

This study was designed by G.M.N., Z.R., N.G.W., and M.B.; eDNA samples were collected and processed by G.M.N.; specimens were collected and identified by N.G.W., G.M., A.M.H., and L.K.; imagery was analyzed by R.P.; bioinformatics and data curation were performed by G.M.N.; statistical analyses were conducted by G.M.N.; figures were created by G.M.N.; G.M.N. led the writing of this manuscript with contributions from all co-authors.

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Ethics Statement

This research was conducted in the Gascoyne Marine Park under permits PA2019-00114-1 and AU-COM2020-476 issued by the Director of National Parks, Australia.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

ROV imagery and annotations are available on Squidle+ through campaign FK200308 (<https://squidle.org/geodata/explore#map>). Cruise data, bathymetry, and related expedition information are accessible via the Schmidt Ocean Institute (<https://schmidtoclean.org/cruise/illuminating-biodiversity-of-ningaloo-canyons/#data>) and AusSeabed (<https://www.ausseabed.gov.au/data>). All data generated and R scripts created in this study have been deposited in a Zenodo repository (<https://doi.org/10.5281/zenodo.18581057>). This includes sampling information, raw sequencing data, morphology data, ZOTU tables for both metabarcoding assays, and results of statistical analyses. Custom DNA reference sequences are available on NCBI (see Table S3 for GenBank accession numbers).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** edn370261-sup-0001-DataS1.xlsx. **Data S2:** edn370261-sup-0002-DataS2.docx.