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Coral monitoring in northwest Australia with environmental DNA metabarcoding using a curated reference database for optimized detection

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Abstract

The need for efficient and more accurate ways of monitoring threatened ecosystems is becoming increasingly urgent as climate change intensifies. Coral reefs are an example of an ecosystem in crisis, with widespread declines in coral cover and diversity documented over recent decades. Novel molecular approaches such as biomonitoring using environmental DNA (eDNA) from seawater samples show great potential to complement future coral reef monitoring programs, especially when used in combination with conventional methods. However, eDNA metabarcoding studies often rely on public databases (e.g., GenBank) for assigning taxonomy, which generally limits the number of sequences that can be taxonomically identified. The extent to which building reference tissue sequences improves taxonomic resolution has yet to be fully examined. Here, we combined traditional coral reef monitoring data with eDNA assessments derived from seawater collected at the highly diverse Rowley Shoals in Western Australia. Using two ITS2 assays developed to target basal metazoan DNA and a reference database spiked with 70 local coral specimens, we identified 37 genera and 40 species from 56 1 L seawater samples. We identified considerable overlap of taxa with visual survey data and showed that assignment of amplicon sequence variants was significantly improved when "spiking" the taxonomic classifier with curated sequences of locally collected species. Our findings showcase the potential of eDNA metabarcoding for monitoring the biodiversity of reef corals and highlight the importance of custom reference sequence databases for improving taxonomic resolution in metabarcoding studies.

KEYWORDS
biodiversity, coral reefs, eDNA, ITS2, metabarcoding, monitoring

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1 | INTRODUCTION

Coral reefs are biodiverse and dynamic ecosystems that are rapidly changing due to anthropogenic activities and climate change (Benkwitt et al., 2020). Severe declines in coral cover and biodiversity have been recorded globally, and increasingly, these declines are linked to prolonged periods of abnormally elevated sea surface temperatures (Descombes et al., 2015; Gilmour et al., 2013; McClanahan et al., 2019; Moore et al., 2012). Such marine heat wave events have caused extensive coral bleaching and mortality over large spatial scales (Gilmour et al., 2019; Hughes et al., 2003, 2017), and there is growing concern that the window of opportunity for recovery between bleaching events is narrowing (Hughes et al., 2018). As a result, there is an urgent need to document biodiversity on reefs to prevent silent extinctions (Richards & Day, 2018), especially as thermal anomalies are predicted to intensify over the coming decades (Ateweberhan et al., 2013; Frieler et al., 2013).

Effective coral reef management relies heavily on the accuracy and comprehensiveness of biodiversity data collection, as well as species richness and distributions (Deiner et al., 2017; Rees et al., 2014). Additionally, monitoring the spatial and temporal variation in coral diversity is required to inform managers of changes in reef community structure (Ryan et al., 2014). Long-term coral reef monitoring programs can help scientists and managers evaluate the state of reefs and increase our understanding of trends at a local scale (Lasagna et al., 2014). Surveys of coral cover and diversity generally rely on diver-based assessments, which can be expensive, logistically difficult, and require a high level of taxonomic expertise (Hill & Wilkinson, 2004). Additionally, surveys that aim to capture changes in coral cover may not adequately document changes in diversity on reefs (Richards, 2013). With over 600 species of scleractinian coral documented in the Indo-Pacific region (Veron et al., 2015), improvements in datasets and monitoring methods are needed to appropriately capture changes in this diversity, especially given the difficulty in applying traditional morpho-taxonomic identifications.

High-throughput sequencing approaches have the potential to revolutionize biomonitoring of highly diverse communities, such as scleractinian corals. Metabarcoding techniques are increasingly being used for biodiversity assessment to detect a range of target species and taxa in various ecosystems (Cilleros et al., 2018; Everett & Park, 2018; O'Donnell et al., 2017; Thomsen et al., 2012). Environmental DNA (eDNA) metabarcoding makes use of the genetic material contained in environmental samples such as air, soil, or water to simultaneously detect a range of amplified and sequenced target DNA (Taberlet et al., 2012). Despite being widely used to monitor species biodiversity and detect target species (Minamoto et al., 2017; West et al., 2020), the application of eDNA metabarcoding to monitor coral diversity remains in its infancy. Scleractinian corals have been successfully detected in seawater using a multi-assay approach with ITS2 and 16S genic regions (Alexander et al., 2020; West & Adam et al., 2021), and their biomass was correlated to the abundance of coral eDNA reads in Hawai'i (Nichols & Marko, 2019). The ability to interpret eDNA metabarcoding data is significantly constrained by the quality and quantity of the reference sequence data (Alexander et al., 2020; West & Adam et al., 2021). Certainly, for many cnidarians, there is currently a paucity of reliable reference sequence data, which limits the full potential of eDNA metabarcoding for coral monitoring to be realized. With gaps in reference databases previously highlighted as hindering comprehensive taxonomic assignments (Leduc et al., 2019; McGee et al., 2019; Shinzato et al., 2018), the extent to which a curated tissue sequence reference database may improve taxonomic resolution has yet to be fully examined.

Here, we examine the application of eDNA metabarcoding as a tool for coral monitoring at the Rowley Shoals, a remote atoll system in northwest Australia. To improve the taxonomic resolution of our eDNA surveys, we curated a reference sequence database using museum voucher specimens for 94 species of coral collected from the Rowley Shoals. We then used a multi-assay ITS2 metabarcoding approach to compare estimates of coral genus richness from seawater samples to those obtained from traditional visual surveys. We collected seawater samples from the surface of the water and at depth, to better understand the importance of sampling location on monitoring shallow water coral biodiversity. Our results highlight the utility of eDNA metabarcoding as an emerging and complimentary tool for monitoring coral biodiversity on tropical reef ecosystems, particularly where a local reference sequence database is available.

2 | MATERIALS AND METHODS

2.1 | Visual surveys

Located within the Oceanic Shoals Marine Bioregion, approximately 260 km from the coastline of northwest Australia, the Rowley Shoals consists of three oceanic reef atolls (Figure 1). Mermaid Reef, Clerke Reef, and Imperieuse Reef are characterized by high water quality, exceptionally rich diversity in near natural state, and are largely unaffected by many of the pressures affecting coral reefs globally (DEC, 2007). Visual surveys were conducted at Clerke and Imperieuse atolls at seven long-term monitoring sites located in the reef slope (6 – 9 m depth), lagoon bommie (< 3 m depth), and lagoon channel (6 – 9 m depth) habitats which differed in coral cover, diversity and assemblage. Depending on the distribution of the community in each habitat, benthic cover was assessed across a single 50 m transect at two sites (IC1 and IL2), across three replicated 50 m transects at one site (CL1), and across five replicated 50 m transects at the remaining sites (CL2, IL1, IS1, IS2). Photographs were taken 30 to 50 cm above the substrata at one-meter intervals, and corals were identified to the genus level using the point intercept method (Jonker et al., 2008).

2.2 | Metabarcoding survey

For each of the sites surveyed in the visual assessments, we collected eight seawater samples in 1 L Nalgene bottles (N = 56), cleaned by soaking for > 10 minutes in a 10% domestic bleach solution. Replicate
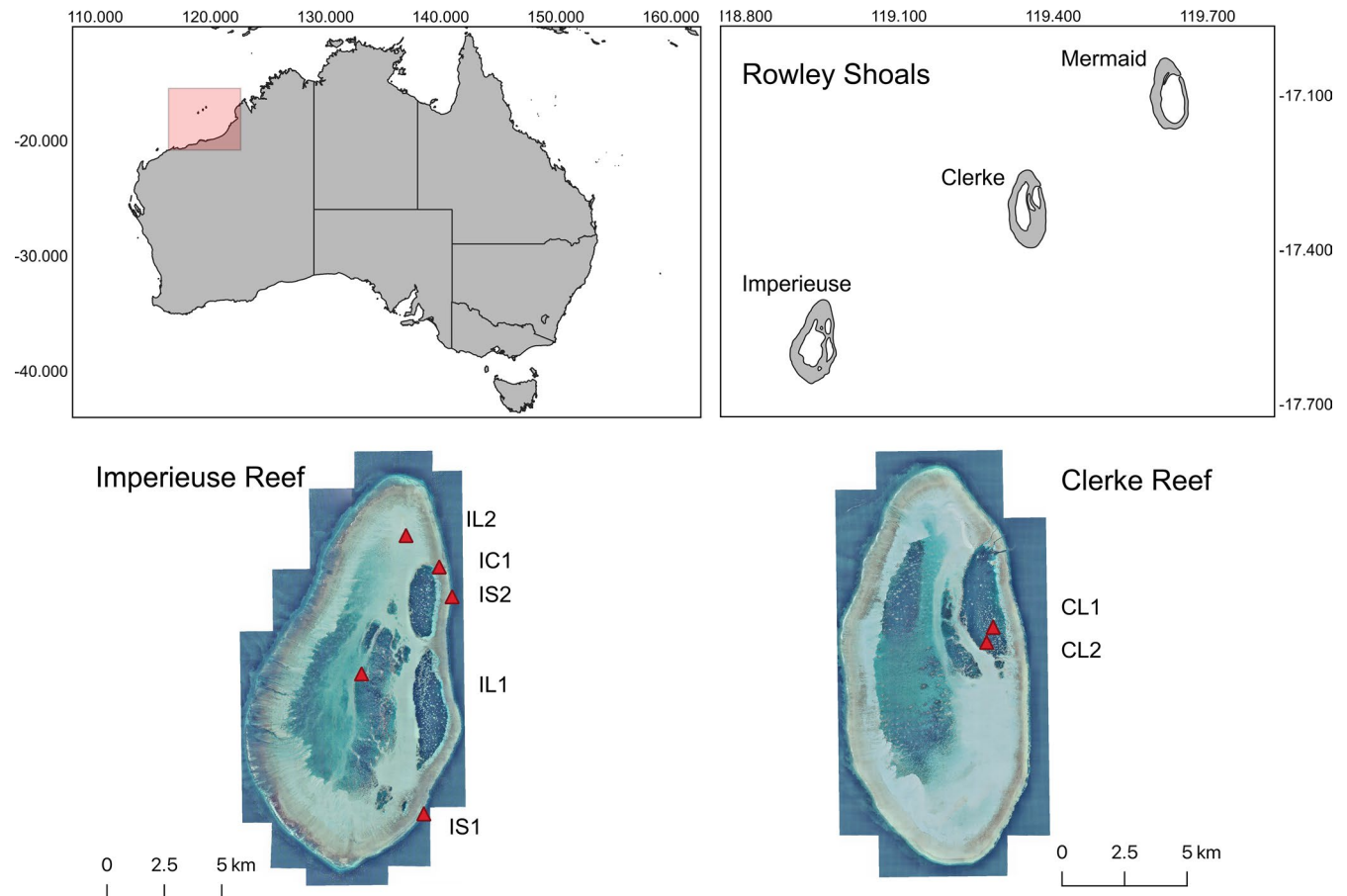
FIGURE 1 Location of the sampling sites within Imperieuse and Clerke reefs at the Rowley Shoals, northwest Australia

samples were collected just below the surface (n = 4) and from just above the substrata (n = 4), approximately 25 m along the first 50 m survey transect. The replicate samples were taken in the same vicinity, within a meter from each other. Water samples were filtered within eight hours of sample collection using a Sentino Microbiology Pump (Pall Corporation). The filtering equipment was sterilized by soaking the pump parts in a 10% domestic bleach solution for at least 10 minutes between processing different samples. Following filtering, the 0.45 µm polyethersulfone membrane filter papers (Pall Life Sciences) were folded and stored in cryogenic tubes at −80°C until processing in a laboratory. Filter papers were cut in half; one half preserved in storage and the other cut in small pieces for DNA digestion using the DNeasy Blood and Tissue kit (Qiagen) with the following modifications to the protocol: 360 µL of ATL buffer and 40 µL of proteinase K for a 3-hour incubation at 56°C. The resulting supernatant was loaded into the QIAcube DNA extraction robot (Qiagen) for automated DNA extraction.

2.3 | Reference tissue sequence material

To improve taxonomic resolution of our eDNA surveys, we curated a reference database using Western Australia Museum voucher specimens for 94 species of coral (39 genera) collected from the Rowley Shoals in 2014 (File S1). Specimens were classified (Z. Richards) to species-level using the AIMS Monograph Series (AIMS, 2017) and according to established methods (Veron, 2000; Wallace, 1999; Wallace et al., 2012; Wells, 1954), the Corals of the World online database and the classification system detailed in the World Register of Marine Species (WoRMS). DNA was extracted from ethanol-preserved tissue samples using the DNeasy Blood and Tissue kit and the QIAcube DNA extraction robot (Qiagen) following the manufacturer's protocol.

2.4 | PCR amplification and sequencing

Two metabarcoding assays (CoralITS2 and CoralITS2_acro; Table S1) targeting the ITS2 gene were used on both the eDNA and reference tissue extracts (Alexander et al., 2020; Brian et al., 2019; West & Adam et al., 2021). Amplicons were approximately 419 bp for CoralITS2 and 445 bp for CoralITS2_acro. While the ITS2 region has proved inefficient for coral phylogenetic reconstruction and poses problems with intragenomic variation (Vollmer & Palumbi, 2004) and multicopy nature, the high level of sequence diversity in Cnidaria makes this genomic gene region an attractive marker for

environmental DNA metabarcoding studies. The CoralITS2_acro assay (modified reverse primer) was used because the ITS2 region of *Acropora* (the dominant coral genera at the study location) is shorter and prohibited efficient primer binding, hence hindering the detection of the genus (Alexander et al., 2020; Chen et al., 2004).

Quantitative PCR (qPCR) was performed in a single step using duplicates of each samples and fusion tagged sample-specific tag combinations (6 – 8 bp long, MID-tag), including Illumina sequencing adaptors, for each assay. Each qPCR reaction was made up to 25 µL and contained: UltraPure Distilled water (Invitrogen), 10x PCR Gold buffer (Applied Biosystems), 50 nM $MgCl_2$ (Applied Biosystems), 0.4 mg/ml bovine serum albumin (BSA; Fisher Biotec), 25 nM dNTPs (Astral Scientific), 5x SYBR Green (Life Technologies), 1 U AmpliTaq Gold PCR buffer (Applied Biosystems), 10 µM of forward and reverse primer, 20 µM of forward and reverse tags and DNA template. PCR conditions were as follows: initial denaturation at 95°C for 5 min followed by 45 cycles of 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 45 sec, with a final extension of 72°C for 10 min. Amplicons for each qPCR run were pooled into Mini Pools (MP) using 10 µl per sample, so that each MP had a ΔRn range of maximum ~5000. Size distribution and DNA concentration of each MP were verified using the OM500 method on the QIAxcel Advanced instrument (Qiagen). MPs were blended to equimolar volume based on QIAexcel-derived concentrations to form a final library for each sequencing run. Three libraries were created, one for the seawater CoralITS2 samples, one for the tissue CoralITS2 samples, and one for the CoralITS2_acro samples (tissue and seawater; see workflow in Figure S1). The final libraries were size-selected to a range of 160–550 bp (for CoralITS2) and 175–600 bp (for CoralITS2_acro) using Pippin Prep (Sage Science, USA) and purified into 30 µL using the Qiagen QIAquick PCR purification kit and protocol (Qiagen). The cleaned libraries were then quantified using fluorometric quantitation (Qubit 4.0, ThermoFisher) and sequenced across three different MiSeq 500-cycle V2 chemistry paired-end runs (Illumina, USA) at the TrEnD lab, Curtin University, Australia.

2.5 | Bioinformatics and Statistical Analyses

For each sequencing library, we used the INSECT package (v.1.1.1.9; Wilkinson et al., 2018) to demultiplex reads based on unique tag combinations, removing sequences with no exact match to the tag sequences. DADA2 (Callahan et al., 2016) was used to trim reads, dereplicate, merge reads, remove chimeras and cluster into Amplicon Sequence Variants (ASVs) (R Script in File S3). ASVs are unique sequence reads separated by single nucleotide differences and are not based on arbitrarily chosen dissimilarity thresholds (Callahan et al., 2016). The LULU algorithm was then used to curate ASV results, as it reduces the number of erroneous ASVs by merging "daughter" ASVs with consistently co-occurring more abundant "parent" ASVs (Frøslev et al., 2017). INSECT is a tool that uses profile hidden Markov models to assign highly accurate taxon IDs to amplicon libraries by taking reference sequences (e.g., GenBank), filtering

and trimming them to the region of interest, and building classification trees. The "classify" function outputs the taxon name, rank and ID number (originating from the reference database), and the Akaike weight value that is used as a confidence score (ranging 0 to 1, with 1 indicating high taxonomic confidence). There are INSECT classifiers for multiple primers, including the ITS2 region used for cnidarians and sponges (Wilkinson et al., 2018). We downloaded metazoan ITS2 sequences from GenBank on 25 July 2019 using the INSECT "searchGB" function. Chordate sequences, those with no species-level ID, and those with organism identifiers containing either *fungal*, *endophyte*, *symbiont*, *unclassified*, *unidentified*, *predicted*, *unknown*, *environmental*, *metazoan*, or *eukaryote* were discarded. The curated database was then trimmed with CoralITS2 and CoralITS2_acro primer sequences using the "virtualPCR" function, retaining only sequences with high scoring hits and length range of 50 to 500 bp. The seawater samples were classified against this database using the "classify" function with Akaike weight confidence threshold of 0.80 (threshold = 0.8), a nearest neighbor minimum identity threshold of 99% (ping = 0.99), and a minimum reference sequence cluster size of 2 (mincount = 2, File S2). In order to reliably develop a reference database of known ITS2 coral sequences, we applied a conservative filtering step by cross-checking coral tissue ASVs with BLASTn to ensure they had at least accurate genus-level assignments matching sequences on GenBank (BLASTn e-value 0.001). This was done to verify that we successfully amplified the target species and not a trace DNA signal originating from within the coral tissue. Indeed, because coral tissue represents a complex environmental sample that can contain traces of multiple species (possible through ingested material) that can differentially amplify under either CoralITS2 or CoralITS2_acro, we performed this critical but strict quality control step despite losing the DNA information from species not retained for the database. All correct ASVs were retained, meaning that for each specimen, multiple ASVs were selected if they were a congener, and these sequences were used to "spike" the database (hereafter referred to as spiked database). The seawater ASVs were then re-classified against the spiked database using the same parameter settings used above (see workflow in Figure S1).

Once taxonomy was assigned to the ASVs from the seawater samples, data from the CoralITS2 and CoralITS2_acro barcodes were compared to identify differences in taxonomic detections. We then aggregated reads belonging to different species within a genus and merged the two assay-specific datasets to create a final abundance matrix of genus-level detections. To allow for the capture of rarer taxa, only singletons were removed from the dataset (Alberdi et al., 2017; Burgar et al., 2014) before transforming the data into a binary matrix to conduct further analyses. We evaluated how eDNA and visual methods surveyed the coral communities using species accumulation curves from the iNEXT package (Chao et al., 2014; Hsieh et al., 2020) and estimated the total richness of the system using the Chao2 estimator in SpadeR (Chao & Chiu, 2014) based on all genus-level detections in the visual survey data. We conducted a Spearman Rank correlation test using "cor.test" to test for a correlation between estimates of genus richness based on visual and eDNA

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methods at each site. To investigate how eDNA detection varied between the surface and substrata depths at each site, PERMANOVA analyses with 999 permutations were conducted using the "adonis" function in the *Vegan* package based on a Jaccard distance matrix (Oksanen et al., 2019) using Bonferroni to correct for multiple testing. Homogeneity of variance was then tested using "permutest" to verify whether differences are due to true differences between groups (depths) or due to dispersion. The intragenomic variability of the ITS2 marker, as seen with the number of correct ASVs recovered in each tissue sample, was visualized using the PopART software (Leigh & Bryant, 2015).

3 | RESULTS

3.1 | Seawater sequencing results

Metabarcoding of 56 seawater samples yielded a total of 8,024,595 demultiplexed sequences (Table 1), with an average of 32 861 reads per sample (Figure S2). Three surface seawater samples failed to amplify during PCR using CoralITS2 and were not sequenced (1S2 at site IC1, 4S2 at site IL1 and 7S2 at site IL2) whereas all seawater samples amplified using CoralITS2_acro. PCR controls (no template control; NTC) showed no sign of successful amplification (within 45 qPCR cycles) and were not sequenced. After LULU curation, we found an average of 131 and 143 ASVs per sample using CoralITS2 and CoralITS2_acro, respectively. Of the 6495 total ASVs recovered across both assays, approximately 55% could be reliably classified to the phylum level or lower using the standard GenBank database (Table 1). Of those, Cnidaria accounted for approximately 53.7% and 84.6% of the total diversity using the CoralITS2 and CoralITS2_acro assays, respectively (Figure 2). After merging ASVs based on identical taxID matches, 109 and 78 unique annotations remained based on CoralITS2 and CoralITS2_acro, respectively. Within Cnidaria, an average of 18.5% (± 4.9) consisted of species-level coral detections (across both assays).

3.2 | Reference material sequencing results

Metabarcoding of the 94 coral specimens yielded a total of 3,120,488 demultiplexed reads across both assays (Table 2). PCR controls (NTC) showed no sign of successful amplification (within 45 qPCR cycles) and were not sequenced. Out of the 94 original specimens,

70 specimens were assigned at least one reference sequence. We observed a range of one to nine correct ASVs per tissue sample, with the majority of specimens (50/83 corals; 60.24%) generating only one ASV retained for the reference database. Some specimens however generated multiple correct ASVs (Figure S3). Overall, the reference sequences comprised of 78 ASVs (from 62 specimens) and 74 ASVs (from 21 specimens) from CoralITS2 and CoralITS2_acro, respectively (GenBank accession MW473514-MW473666). For the remaining 24 specimens (out of 94), none of the ASVs generated in each sample matched to a congener on GenBank. These could not be reliably integrated into the reference material due to the lack of confirmation that the ASV sequence generated via metabarcoding corresponded to the specimen (Table S6).

3.3 | Impact of developing a reference tissue sequence database

The spiked database contained 125 and 85 annotations using CoralITS2 and CoralITS2_acro, and within Cnidaria, an average of 33.1% (± 3.5) consisted of species-level detections (across both assays). For both assays, the number of coral ASVs annotated at the family, genus, and species level increased considerably, showing an overall improved resolution across the different taxonomic levels (Figure 3). Using CoralITS2, 10 genera recovered using the spiked database were not detected by relying solely on standard GenBank sequences and using CoralITS2_acro, two genera were gained using the spiked database. Collectively, the two assays generated 37 unique genera (29 hard corals and eight soft corals), as well as 40 species using the spiked database (Table S5). This constituted a significant increase from the 30 unique genera (23 hard coral and seven soft coral) and 17 species found in the dataset resulting from standard GenBank sequences. Additionally, the number of species within 13 different genera increased when using the spiked database, resulting in finer species-level resolution across a range of coral taxa. Over 67% of the genera in the spiked dataset could be assigned at least one species, a ~ 21% increase from standard GenBank sequences.

3.4 | Assay overlap and sampling depth

There was considerable cross-amplification of genera across the two assays, but some groups were missing from one dataset (Figure 4).

TABLE 1 Number of sequences, reads per seawater sample, and ASVs that remained after each bioinformatic step of the DADA2 and LULU pipelines for the two MiSeq sequencing runs

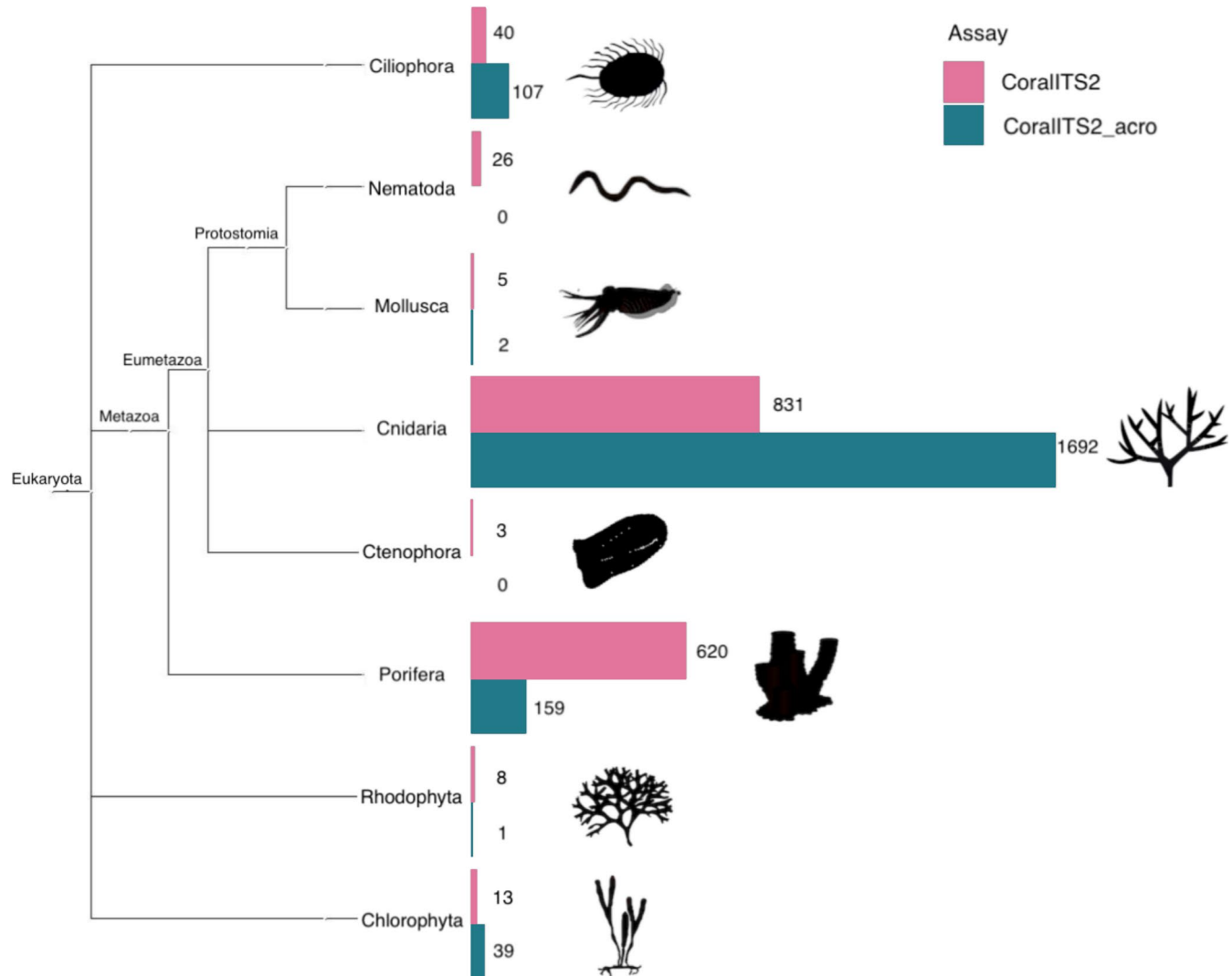| Sequencing run	Demultiplexed	Quality filtered	Merged	Chimera-free	Reads per sample	DADA2 ASVs	LULU ASVs	Annotated ASVs
CoralITS2	6,035,076	5,624,403	5,275,530	4,815,664	90,861 (SE ± 29,120)	5114	2499	1546
CoralITS2_acro	1,989,519	1,653,506	1,561,639	1,454,716	25,977 (SE ± 11,061)	4253	3996	2000

FIGURE 2 Phylum-level diversity recovered in eDNA samples. Taxonomic tree indicating the number of ASVs recovered in the eight phyla detected with CoralITS2 and CoralITS2_acro assays

TABLE 2 Number of sequences, reads per sample, and ASVs that remained after each bioinformatic step of the DADA2 and LULU using reference tissue material

| Sequencing run	Demultiplexed	Quality filtered	Merged	Chimera-free	Reads per sample	DADA2 ASVs	LULU ASVs
CoralITS2	889,868	801,285	738,781	679,717	7308 (SE ±3791)	535	247
CoralITS2_acro	2,230,620	1,682,569	1,596,487	1,295,446	32,386 (SE ± 18,831)	505	101

CoralITS2 recovered 15 unique genera, and CoralITS2_acro recovered three unique genera, showing that while using only CoralITS2 may be sufficient to recover the majority of corals in our study system, a few groups (notably, *Acropora*) would not be represented by using only one assay (Figure 4). Indeed, the *Acropora* genus is ecologically important in our study system due to the reefs being *Acropora*-dominant, and the lack of CoralITS2 detection confirmed our initial expectation and the need for a multi-assay approach. Using this spiked dataset of merged detections across the two assays, we found no effect of sampling depth on the eDNA compositions recovered at the seven sites

(PERMANOVA; Table S2; Figure 5), indicating that eDNA signals are generally homogeneous throughout the shallow water column (< 9 m in depth). Homogeneity of variance tests also suggested homogeneous dispersion within each site (Table S2).

3.5 | Comparison with visual surveys

Across the seven sites, visual surveys recorded a total of 42 genera, comprising of 36 genera of hard coral and six genera of soft

FIGURE 3 Barplot showing differences in the number of ASVs detected at different taxonomic levels using the standard GenBank and spiked databases for both the CoralITS2 and CoralITS2_acro assays
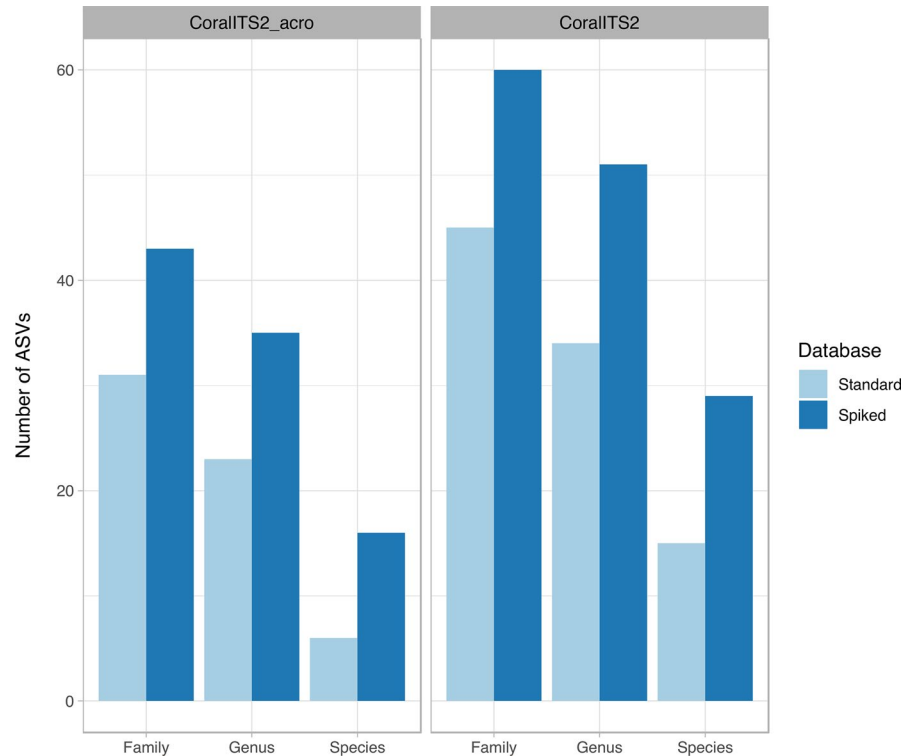
coral (Table S3). Combined with the 37 genera detected with metabarcoding, a total of 56 unique genera of coral were recovered. Approximately 43% of the total diversity detected using seawater eDNA was also recorded in the visual surveys. The visual surveys captured a slightly higher number of unique taxa than metabarcoding surveys (Figure 6C). Within sites, the number of genera detected was higher for metabarcoding than visual surveys for two of the seven sites, but at both of these sites, visual surveys were restricted to a single 50 m transect because of the patchy distribution of the coral assemblage (Figure 6B, Table S4). The overlap in the number of genera common to both methods ranged from 15 to 40.6% with an overall average of ~31.5% (± 3.6; Table S4). Although we identified considerable taxonomic overlap between methods, we did not identify a positive correlation between genus richness identified by the two methods at the site level (Spearman Rank test, p = 0.682, r = 0.191). For example, one site (IL2) was sampled from a large sand patch mostly void of any coral cover and located more than a kilometer away from species rich areas. At that site, three genera were detected by visual surveys, 20 genera were recovered with eDNA, and three were shared between methods (Figure 6B). Similarly, at the Imperieuse channel site (IC1) with low diversity (dominated by *Isopora brueggemanni*), eDNA metabarcoding detected more than double (n = 24) the genus richness recovered via visual surveys (n = 9).

Species accumulation curves calculated across all samples indicated that the coverage of gamma diversity was adequate for both methods (Figure 7). Based on the sampling effort used for the different methods, the diversity recovered using visual surveys (25 x 50 m transects) plateaued earlier than with metabarcoding surveys (56 x

1L seawater samples). According to the Chao2 index (SpadeR; Chao & Chiu, 2014) calculated from the visual survey dataset, total richness of the system was estimated at 48 coral genera (CI: ~43 – 74). Metabarcoding and visual surveys detected approximately 77.1% and 87.5% of the estimated total richness, respectively. Capturing the total estimated richness would require approximately twice the sampling effort for both methods (Figure 7).

4 | DISCUSSION

In this study, we explored the use of eDNA metabarcoding as a coral biodiversity monitoring tool on a remote and highly diverse atoll system in northwest Australia. Our survey recovered a total of 6495 ASVs, 53.7% and 84.6% of which belonged to Cnidaria using the CoralITS2 and CoralITS2_acro assays, respectively. Developing a reference sequence database of locally collected coral tissue samples resulted in a 2.3 fold increase in the number of species detected, compared to taxonomic assignments based on a standard GenBank reference database. Our data also showed considerable overlap in coral identification between traditional visual survey and eDNA metabarcoding data, with a 43% overlap in the genera identified. We also found no differences in the coral assemblages recovered in the bottom and surface seawater samples collected at the seven sites, indicating that sampling could be simplified to collection from a vessel. Overall, our results highlight the utility of eDNA metabarcoding as an emerging and complimentary tool for monitoring coral biodiversity on tropical reef ecosystems, particularly where a local reference sequence database is available.
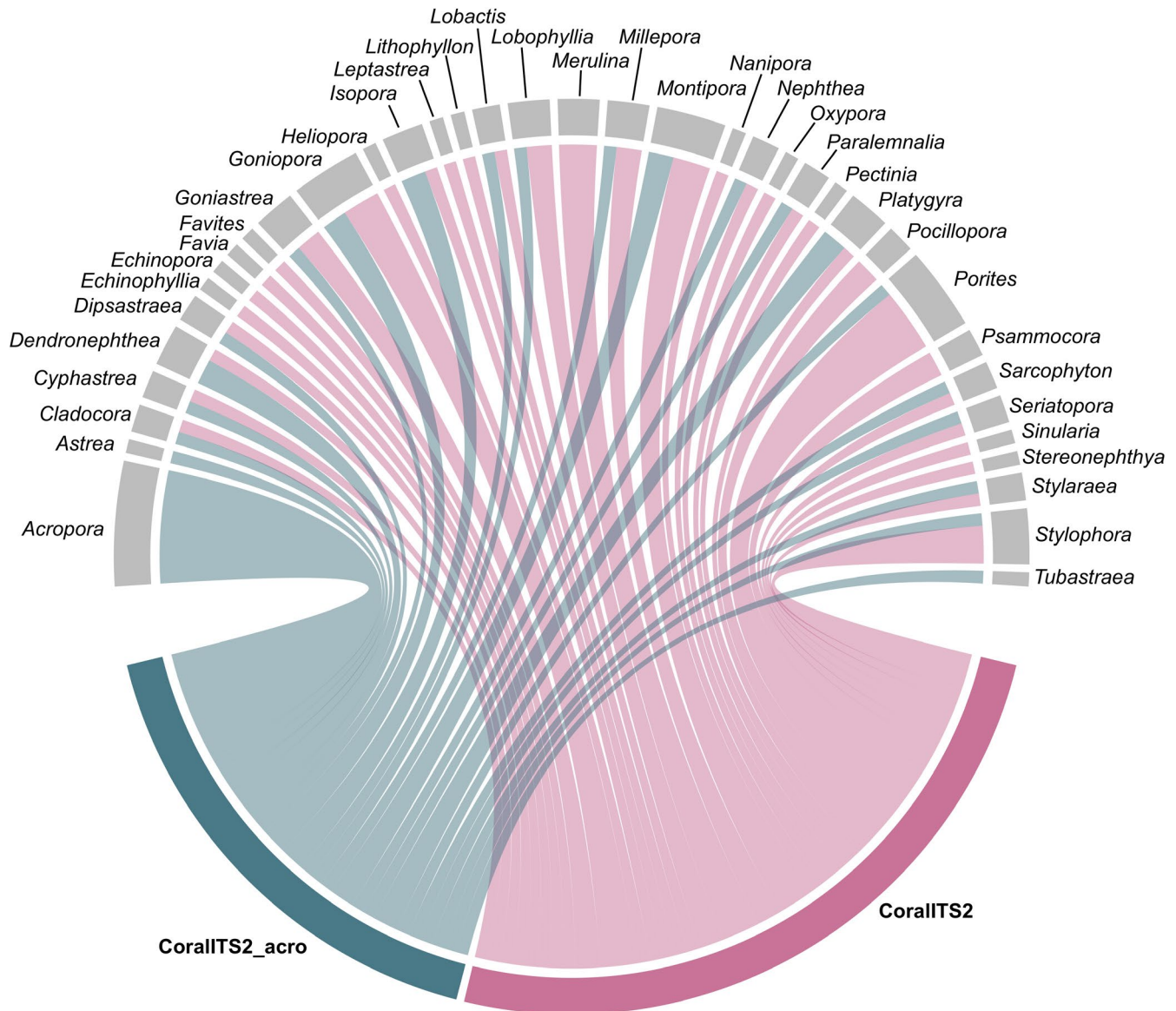
FIGURE 4 Genus-level chord diagram of coral detections across the two assays and using the spiked dataset. Size of endpoints corresponds to the number of unique ASVs attributed to each genus

4.1 | Locally sourced reference database improves taxonomic resolution

Although the taxonomic reliability of GenBank has been examined and confirmed (at least for metazoan mitochondrial sequences; Leray et al., 2019), an important bottleneck hindering the assignment of taxa from environmental samples is incomplete reference databases (Elbrecht et al., 2017; Leduc et al., 2019; McGee et al., 2019). Our findings confirm that the performance of coral eDNA metabarcoding is influenced by the quality of reference sequences and that developing locally curated databases can considerably improve taxonomic resolution (Zinger et al., 2019). In this study, using a reference sequence database spiked with local sequences yielded more than double the number of species-level annotations and seven extra genera. This step is especially valuable for scleractinian corals, for which reference sequences

are severely lacking (Alexander et al., 2020; Shinzato et al., 2018). Incomplete coral DNA databases impeded precise species identification of 19 *Acropora* species in a mesocosm experiment (Shinzato et al., 2018). Similarly, an eDNA survey yielded very few assignments for Guianese fish taxa when using GenBank for annotations (11% of all reads), whereas using a custom database of 193 references sequences resulted in 46% of all reads annotated (Cilleros et al., 2018). Developing quality reference databases to support eDNA-based detections will be increasingly important as the field of eDNA metabarcoding progresses (McGee et al., 2019). The ongoing development and expansion of reference databases are needed to adequately capture population-level variation within species, for example by sequencing specimens from various regions. Additionally, when using multicopy markers such as ITS2, intragenomic variability should be represented and accounted for when building reference sequences using single-source material.

FIGURE 5 Non-metric multidimensional scaling (nMDS) ordination plot of coral communities recovered in the bottom (above reef) and surface samples (sea surface). This nMDS is based on presence–absence data converted to a Jaccard dissimilarity matrix
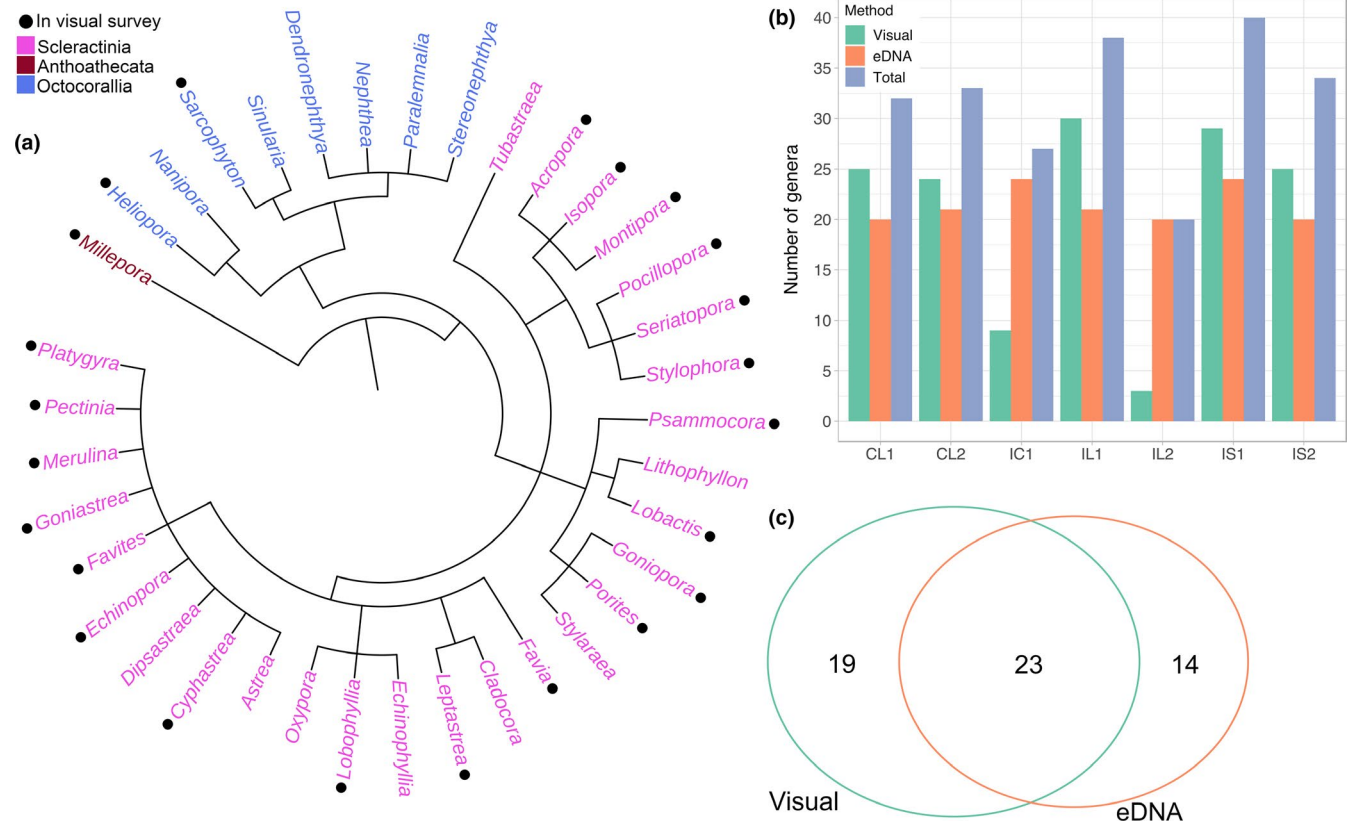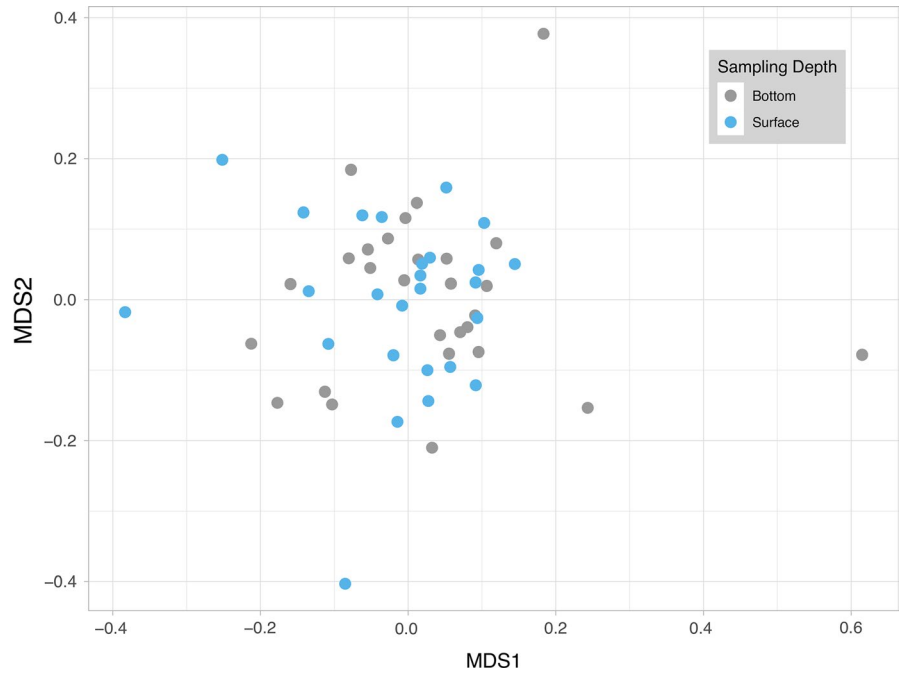

FIGURE 6 Comparison of genus-level coral diversity recovered by eDNA and visual surveys. (a) Genera detected with combined CoralITS2 and CoralITS2_acro assays. Black dots indicate the genera also found with the visual surveys (*Lobactis* was recorded as *Fungia* in visual surveys). (b) Number of genera detected at each site using both approaches individually, and the total number with a combined approach. (c) Venn diagram depicting the unique number of genera identified using both approaches, and the number of genera overlapping between the datasets
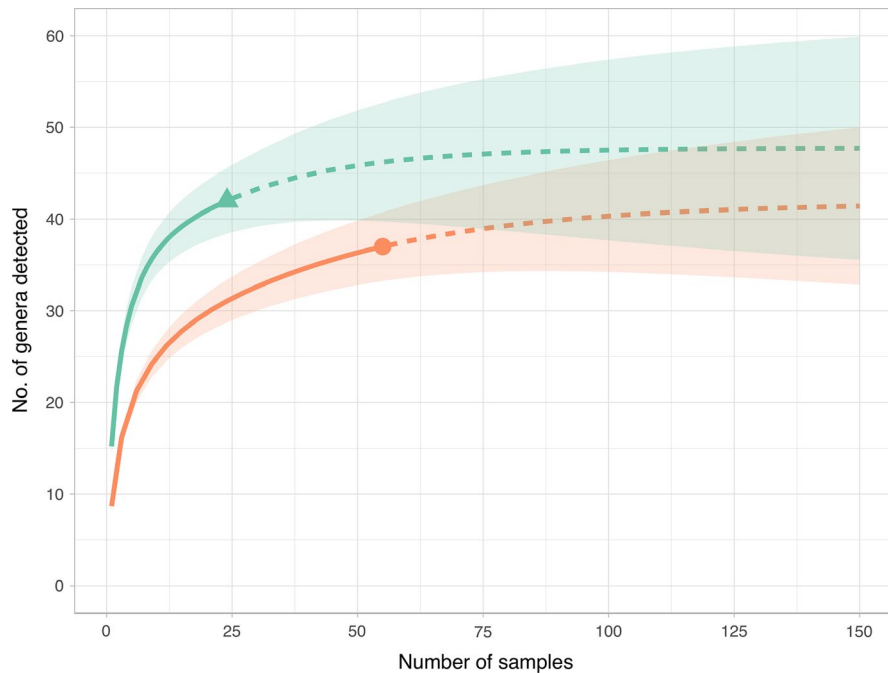
FIGURE 7 Species accumulation curve. Computed with iNEXT and hill number q = 0 (observed richness) using coral diversity estimated by visual surveys (green with triangle) and by metabarcoding (orange with circle). Dotted line represents SAC if sampling effort was increased, extrapolated to 150 samples. Shading shows the 95% CI based on unconditional SD

4.2 | Complementarity of eDNA and traditional methods for coral assessments

Seawater eDNA metabarcoding revealed comparable patterns of coral diversity when compared to traditional visual surveys. Approximately 43% of the genera detected were shared between both methods; however, our eDNA surveys failed to detect four genera (five species) recorded using visual surveys, even when the database was spiked with local sequences (Table S7). For example, *Hydnophora*, *Scapophyllia*, and *Galaxea* were not detected with eDNA despite local reference sequences (*H. rigida*, *H. exesa*, *S. cylindrica*, and *G. fascicularis*) in the database. It is possible that the DNA concentration from these rarer species (< 1% of total cover) was too low to be consistently recovered in environmental samples (Table S3). However, *Pavona* was also absent from eDNA detections (*P. explanulata* reference sequence in the database), despite contributing up to 5.7% of coral cover at some sites in this study (Table S3), and its previous detection using both assays at the Cocos Keeling Islands (Alexander et al., 2020) and the Kimberley (West et al., 2021). Low copy number of eDNA templates and primer mismatches can lead to amplification biases (Stat et al., 2018) because taxa can be outcompeted in a pool of multiple preferably amplified DNA templates. These false negative detections may also be due to insufficient biological replicates (e.g., water samples), technical replicates (e.g., PCR replicates), or sequencing depth (Alberdi & Gilbert, 2019; Willoughby et al., 2016). Increasing technical replicates could counteract issues associated with the differential binding ability of sequences and increase total richness recovered, as well as help to confirm uncertain detections (Alberdi & Gilbert, 2019; Ficetola et al., 2015); however, the strategy used is often a trade-off between keeping true biologically sound sequences and the ability to remove erroneous or artifactual sequences (Alberdi et al., 2017).

Additionally, environmental factors, such as tides and currents, can affect the degradation and transport of DNA (Deiner et al., 2017; Foote et al., 2012). The circulation and residence time of reef waters are important physical processes affecting coral reefs (Lowe & Falter, 2015) and also influence the detection probability of eDNA. Although a considerable overlap was found between the visual and eDNA datasets, within sites there were consistent differences in the number of genera recovered. The detection of 20 genera at IL2, a sandy lagoon site where there was very low coral diversity (only three genera observed) and more than a kilometer away from any diverse assemblage, reflects the extent of mixing and circulation within the lagoon. Similarly, there was a high diversity of genera detected with eDNA metabarcoding at the channel site (IC1) dominated by one species (*I. brueggemanni*), reflecting the flow of water (1 m/second) from the adjacent lagoon, reef crest, and reef slope habitats. At these two sites, the genus richness detected via metabarcoding was higher than with visual surveys, showing that water traveling over distance of a few tens of meters (IC1) to over a kilometer (IL2) can retain a sufficient detectable concentration of eDNA.

A higher percentage of the Chao2-estimated richness of the study sites was recovered with visual surveys using about half the sampling effort than with metabarcoding. This could be explained by the exceptionally high diversity of the system, meaning that less samples are needed to capture a comprehensive overview of coral diversity. However, the inconsistent sampling effort conducted with visual surveys is likely to have impacted the amount of diversity detected across the sites. In two of the seven sites, the genus richness detected via metabarcoding was higher than with visual surveys, although these sites were only surveyed along one 50 m transect due to the patchiness of coral cover. Additionally, visual surveys can be biased toward corals easily visible from above and can fail to record hidden or cryptic species, and corals found just outside

of the transect tape (Nichols & Marko, 2019; Pearman et al., 2016). It should also be noted that the point intercept method is usually deployed to generate benthic cover data rather than biodiversity data; hence, this visual survey approach has clear limitations and likely underestimated the overall richness. Indeed, percent live coral cover is a widely used metric in coral biomonitoring, especially as a way to inform on the condition of coral reefs (Richards & Day, 2018); however, it has been found to be a poor proxy for coral diversity (Richards, 2013; Richards & Day, 2018; Ryan et al., 2014). In this case, the overall higher diversity recorded using a combined multidisciplinary approach showcases the value of integrating eDNA metabarcoding into monitoring programs to capture a more comprehensive snapshot of the local coral diversity (Cilleros et al., 2018; Everett & Park, 2018).

4.3 | No differences between surface and bottom samples

Despite the expectation that eDNA signatures from sessile benthic organisms such as corals may be more easily detectable in samples collected lower in the water column, sampling depth had no effect on the eDNA assemblages recovered. This indicates that on shallow reefs (< 9 m) at the Rowley Shoals, eDNA seems to be homogeneously distributed in the water column with no apparent stratification; hence, sampling could be simplified to collection of seawater samples from the surface. We presume that the similar taxonomic profiles across shallow depths may reflect coral mucus being constantly released (Wild et al., 2004b), often dominating suspended matter around reefs (Johannes, 1967; Marshall, 1968). The majority of this mucus usually dissolves in the water, but a fraction can form floating gel-like mucus aggregates with positive buoyancy (Wild et al., 2004a). However, the residence time of this coral mucus (thus, eDNA) is thought to be low as aggregates trap particles and therefore decrease in buoyancy over time and sink (Wild et al., 2004a). Due to this continuous cycle, sedimentation is estimated to occur within a few hours of release (Wild et al., 2004b). This would indicate that detections from sampling seawater provide a relatively instantaneous snapshot of the coral diversity present at the sampling location. This has important implications for biodiversity monitoring as it demonstrates that changes in coral species richness could be detectable over short timescales.

4.4 | Anomalous detections with eDNA

The coral taxa detected in the seawater samples were consistent with known species records of the region (Long & Holmes, 2009; McKinney, 2006; Richards et al., 2015; Veron & Marsh, 1988). There were, however, some discrepancies. Three species (*Goniopora gracilis*, *Montipora patula* and *Montipora verrilli)* are known to occur in the Western Indian Ocean or Indo-Pacific

region (Sheppard, 1987), and while their presence may be plausible, it requires further validation. Furthermore, we detected two octocorals that are far outside of their known geographical ranges. The detection of the azooxanthellate octocoral *Nanipora kamurai* (100% match to NCBI Accession no. KP195282.1), historically only recorded in Japan and Thailand, is interesting; the colonies typically occur in low abundance in downward orientation or on dead coral skeletons; hence, it is plausible that their discovery has yet to be confirmed in the Central Indo-Pacific region due to their small size and cryptic habitat (Miyazaki & Reimer, 2015; Miyazaki et al., 2016; Urgell Plaza et al., 2017). Secondly, we recorded *Cladocora* sp. (100% match to NCBI Accession no. AY722752), and although the genus is generally found throughout the Caribbean (Baron-Szabo, 2005), and *C. caespitosa* is endemic to temperate Mediterranean waters, the Taiwanese *Cladocora* sp. record (Chen et al., 2004) suggests its distribution may be broader than previously thought. Subsequent confirmation with alternative metabarcoding assays, as well as consistent field and laboratory controls, would provide a more robust assessment of potentially rare or spurious species records. However, these records, while unconfirmed, indicate that eDNA may offer a way to detect cryptic and/or rare species and inform on the biographical ranges of such species.

5 | CONCLUSION

Scleractinian corals are an exceptionally diverse taxonomic group, and our traditional understanding of their taxonomy based on morphology and phylogeny is increasingly being challenged (Huang et al., 2011, 2014; Richards & van Oppen, 2012; Veron, 2013; Wolstenholme, 2004). Indeed, uncertainties in the evolutionary relationships among species warranted a significant number of systematic updates (Fukami et al., 2008; Kitano et al., 2014; Wallace et al., 2012). This may impact and emphasize discrepancies between molecular and morphology-based identifications of corals and limit the integration of both datasets. The combination of methods (eDNA metabarcoding and traditional surveys) resulted in higher overall levels of diversity detected, as has been seen in many other marine eDNA studies (Everett & Park, 2018; Nguyen et al., 2019; Stat et al., 2018). Additionally, both methods detected a substantial number of unique taxa, further indicating that different survey methods have advantages and should most likely be used in conjunction to capture the most representative snapshot of a system, especially in highly diverse environments. The future of coral reef management will likely benefit from integrating eDNA metabarcoding for high-frequency and high-resolution monitoring, especially as anthropogenic pressures continue to impact these ecosystems.

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AUTHOR CONTRIBUTIONS

LD, LT, and JPG designed the study. LT, JPG, and NR undertook field sampling, and ZTR provided samples from the Western Australian Museum scleractinian coral collection. JPG and NR analyzed visual survey data. LD undertook laboratory work, data analysis, and drafting of the manuscript. All authors contributed to the analyses and writing of the manuscript.

DATA AVAILABILITY STATEMENT

Raw sequence data (.fastq files), sample information for the 94 coral species, and R scripts are available at Zenodo.org (https://doi.org/10.5281/zenodo.4596305). The Rowley Shoals ITS2 coral database sequences are available on NCBI under the accession MW473514 to MW473666.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.