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The applicability of eDNA metabarcoding approaches for sessile benthic surveying in the Kimberley region, north-western Australia

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Abstract

The application of environmental DNA technologies is a promising new approach to rapidly audit biodiversity across large-scale, remote regions. Here, we examine the efficacy of a dual-assay eDNA metabarcoding approach for sessile benthic bioassessments in the turbid waters of the Lalang-garram marine parks, Kimberley, north-western Australia. We ask three principal questions: (1) "Is the eDNA released by sessile benthic taxa (i.e., hard and soft corals, sponges and tunicates) locally detectable?", (2) "What level of taxonomic resolution is afforded by eDNA metabarcoding using the ITS2 region?", and (3) "How well does eDNA metabarcoding compare to conventional benthic survey techniques?". We report that a dual-assay eDNA metabarcoding approach can detect approximately 70% of the local benthic taxa (i.e., at a species, genus level). It is, however, not as effective at the individual/population level, detecting only approximately 40% of unique amplicon sequence variant (ASV) signals released by an array of individual benthic organisms at the surveyed locations. In examining the efficacy and resolution of the applied ITS2 metabarcoding markers for bioassessments, we report large gaps in the variety of publicly available benthic ITS2 reference sequence data, limiting our ability to provide robust taxonomic assignments. These findings highlight the need to extend ITS2 databases for greater regional representation. Until this is adequately addressed, we recommend that investigating taxonomic assignments to a genus level is the most robust approach for benthic monitoring using eDNA. Lastly, we found eDNA metabarcoding and conventional belt transect surveys each detected numerous unique hard coral genera, indicating that a combined approach provides the most effective way to audit benthic biodiversity. This point notwithstanding, eDNA metabarcoding had the power to distinguish similar diversity trends between sites to that determined by the belt transect methodology, validating the application of eDNA metabarcoding as either a stand-alone, or complementary technique for assessing sessile benthic taxa.

Katrina M. West and Arne A. S. Adam are equally contributed.

KEYWORDS
benthic biodiversity, biomonitoring, marine invertebrates, marine park, Scleractinia

1 | INTRODUCTION

Species richness influences ecosystem functioning, resilience, and resistance to environmental change. However, for most marine communities, there is a critical shortage of rigorous species-level baseline data which presents a major challenge for the conservation of diversity (Balmford et al., 2005; Richardson & Poloczanska, 2008). For reef-building corals, the challenge of collecting species-level data is exacerbated by the high level of expertise needed to identify corals and assemble reliable biodiversity datasets. In response to this, the need has arisen to develop proxy metrics that accurately represent trends in biodiversity (Baillie et al., 2008). On coral reefs, "reefscape proxies" are commonly used to quantify the condition of coral reef habitat, with percent live hard coral cover being the most widely used metric. Despite its popularity, preliminary studies suggest hard coral cover is not a robust indicator of coral biodiversity (Richards, 2013; Richards & Hobbs, 2014). This is because hard coral cover is a poor linear predictor of coral species richness; thus, a reef with high coral cover does not necessarily have high coral species diversity. As such, there is a need to further optimize the data collected in coral reef monitoring programs, and to find effective alternatives to determine the status of coral biodiversity.

A promising new approach to audit biodiversity and to undertake marine biomonitoring is through the application of environmental DNA metabarcoding technologies. Environmental DNA (eDNA) describes the traces of animal or plant DNA naturally shed into the environment through the loss or excretion of skin cells, mucous, blood, or gametes (Taberlet et al., 2012). Environmental DNA metabarcoding has been successfully used to profile community diversity and compositions, including disturbed and depleted populations (Bakker et al., 2017; Boussarie et al., 2018; DiBattista et al., 2020). In marine ecosystems, eDNA metabarcoding is most commonly undertaken on water, sediment, or gut samples (Berry et al., 2017; Koziol et al., 2018; Port et al., 2016; Takahashi et al., 2020; Thomsen et al., 2012). An ever-increasing body of work is examining the power and limitations surrounding eDNA as a tool for marine surveys. To date, the influence of tides, oceanic movement, depth stratification, and spatial scales on site and habitat discrimination (Jeunen et al., 2019, 2020; Lafferty et al., 2020; West et al., 2020) have been examined. These studies generally concur that eDNA metabarcoding is highly localized and is a promising survey tool that can complement existing survey techniques (such as visual surveys, settlement plates, BRUVS), so long as care is taken during the sample capture and preservation stages.

For benthic invertebrate taxa, including scleractinian corals, the use of eDNA metabarcoding is in its infancy. The first study, undertaken on a Hawaiian coral community using water collected from 2- to 4-m depth, indicated the potential for coral cover to be estimated from eDNA read abundance (Nichols & Marko, 2019). The second study examined replicated surface water collections and revealed fine-scale spatial differentiation in coral assemblages at a diverse and isolated atoll reef system (Cocos (Keeling) Islands; Alexander et al., 2020). This study, which used visual data to ground-truth the eDNA metabarcoding results, indicated that a multi-assay approach was necessary to increase the robustness of scleractinian coral detections. It was concluded that the lack of species-level genomic reference material precludes species-level taxonomic assignments at present (Alexander et al., 2020). Hence, while it has been suggested that eDNA can inform marine spatial planning decision-making (Bani et al., 2020), further studies are needed to examine the level of taxonomic resolution that can be reliably obtained using eDNA and the viability of eDNA metabarcoding for coral biomonitoring.

The inshore Kimberley, NW Australia, provides an ideal model system to examine the applicability of eDNA biomonitoring. It is one of the most biologically significant regions of the world (Wilson, 2014), featuring extensive and internationally significant coral communities (Richards et al., 2018; Richards et al., 2015, 2019). However, the coral communities are difficult to monitor due to a combination of dangerous conditions (turbid water, extreme tidal amplitudes, and the presence of saltwater crocodiles). The Kimberley is also the traditional homeland of numerous native title groups (Austin et al., 2019). In recognition of the need to protect the natural and cultural heritage of the Kimberley saltwater country, six coastal marine parks have been established across the Kimberley over the last decade (North Kimberley, three Lalang-garram marine parks, Yawuru Nagulagun/Roebuck Bay, and Eighty Mile Beach).

The Lalang-garram marine parks are a group of three marine parks beginning ~ 150 km north of Derby. The Dambimangari people's Native Title determination overlies the group of marine parks (herein referred to as the Lalang-garram marine parks). The parks include outstanding geological features such as Yowjab (Montgomery Reef) and occurs adjacent to other important geological features such as Turtle Reef in Talbot Bay (Wilson et al., 2011). Coral reefs are distributed across the marine parks in addition to across in the wider inshore Kimberley region. The status of corals is a key performance measure in the Lalang-garram marine parks Management Plans, so too is the implementation of a cost-effective marine monitoring methodology. Here, we examine the efficacy of a dual-assay eDNA metabarcoding approach for sessile benthic bioassessments in the Lalang-garram marine parks and the turbid inshore waters of the wider Kimberley region. Firstly, we examine the detectability of sessile benthic eDNA by quantifying the detection rate of locally sampled and sequenced benthic invertebrates in collected seawater. Secondly, we examine the level of resolution that at present can be gleaned from eDNA metabarcoding of sessile benthic taxa. Thirdly, we compare eDNA-derived hard coral detections with that of belt and point intercept transects measured at each site, to examine the

efficiency of eDNA metabarcoding as a stand-alone and/or complementary tool to conventional survey techniques. Taken together, this paper seeks to assess the overall applicability and integration of eDNA metabarcoding as a bioassessment tool for sessile benthic taxa, particularly for the use in remote north-western Australia.

2 | METHODS

2.1 | Field sampling

For this study, seven intertidal reef sites were surveyed in Lalang-garram marine parks of the Southern Kimberley region in October 2018 (Figure 1). At each site, 10 × 500 ml surface water replicates were sampled in addition to the opportunistic collection of 1–16 tissue specimens from sessile benthic organisms (Table S1), such as hard corals (order Scleractinia), soft corals (order Alcyonacea), sponges (phylum Porifera), and tunicates (subphylum Tunicata) (Table S4). Tissue specimens were collected at spring low tide and stored in 100% ethanol. Water samples were individually filtered through 0.45-µm cellulose filter membranes using a Pall Sentino® Microbiology pump (Pall Corporation, Port Washington, USA) within 4–6 hr of collection. Between the filtration of each replicate, all filtering equipment was soaked in 10% bleach for a minimum of 10 min and further rinsed with desalinated and filtered water. This was to prevent cross-contamination of eDNA between replicates and sites. Two bleach and desalinated tap water samples were taken at the end of each filtering day to serve as filtration controls.

Additionally, hard coral (order Scleractinia) biodiversity was recorded on three replicate 15 m × 1 m belt transects at each of the seven intertidal reef sites (at low tide), to serve as a detection comparison to the eDNA data. Hard coral percentage was also recorded on three replicate 25-m point intercept transects (100 points per transect) at each of the seven intertidal reef sites.

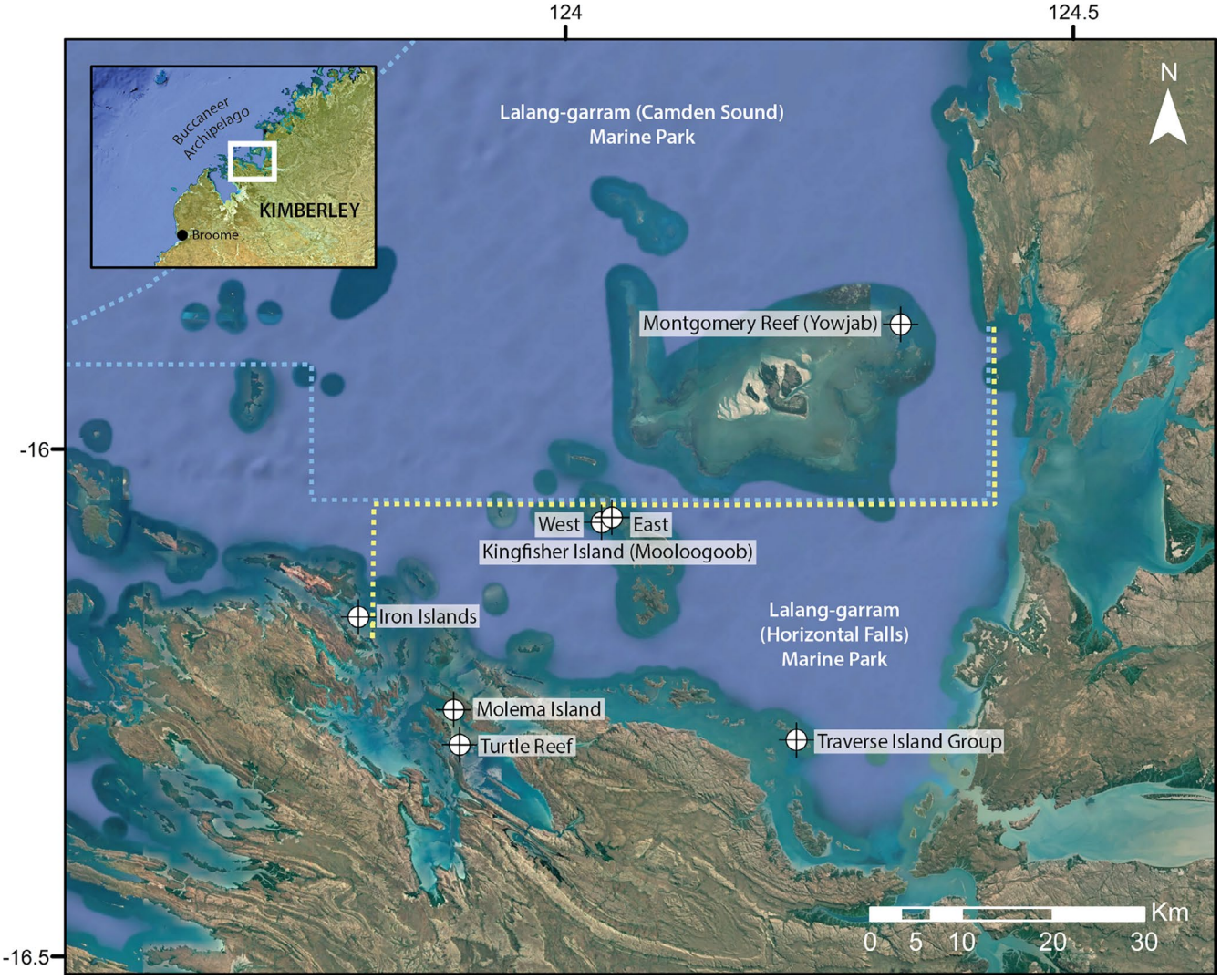

FIGURE 1 Location of sampling sites ($n = 7$) in the Lalang-garram marine parks, Southern Kimberley. Ten × 500 ml seawater samples and sessile benthic organisms were sampled at each site (see Table S1 for more information). The blue dotted line outlines the Lalang-garram (Camden Sound) Marine Park, while the yellow dotted line outlines the Lalang-garram (Horizontal Falls) Marine Park. Map data: Google Earth, SIO, NOAA, U. S. Navy, NGA, GEBCO; image: Landsat/Copernicus

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2.2 | Laboratory processing

Seawater-borne DNA was extracted from half of each respective membrane (including filtration controls) using a DNeasy Blood and Tissue Kit (Qiagen; Venlo, The Netherlands) following the manufacturer's protocol and additional modifications as described in Alexander et al. (2020). Benthic tissue samples were also extracted using a DNeasy Blood and Tissue Kit; however, with the following modifications: 270 µl of buffer ATL and 30 µl of proteinase K were added to each tissue sample and incubated at 56°C for at least 12 hr prior to the remainder of the DNA extraction procedure. Seawater and tissue DNA were eluted off silica membranes in 100 µl and 200 µl of buffer AE, respectively. Each round of daily extractions contained an extraction control. DNA extracts were then stored at −20°C.

Two PCR metabarcoding assays targeting the nuclear ribosomal internal transcribed spacer 2 (ITS2) region were employed for this study: CoralITS2 (Brian et al., 2019) which amplifies a range of scleractinian taxa (exempting the genus *Acropora*), and CoralITS2_acro (Alexander et al., 2020) to amplify *Acropora* (Table 1). The two assays have additionally been found to amplify Actiniaria, Zoantharia, Alcyonacea, and Porifera (Alexander et al., 2020). Quantitative PCR-based (qPCR) quantification was used to screen the quality and optimize levels of input DNA with the following dilutions: 1/5, 1/10, and 1/100 for seawater extracts and 1/500, 1/1000, and 1/2000 for benthic tissue extracts. Each qPCR was carried out in 25 µl containing the following concentrations: 1X AmpliTaq Gold® PCR buffer (Life Technologies, Massachusetts, USA), 2 mM $MgCl_2$ (Fisher Biotec, Australia), 0.4µM dNTPs, 0.1mg BSA (Fisher Biotec, Australia), 0.4 µM each of forward and reverse primers (Integrated DNA Technologies, Australia), 0.6 µl of 5X SYBR® Green (Life Technologies), 1U AmpliTaq Gold® DNA Polymerase (Life Technologies), 2 µl of eDNA template (at optimized dilution), and made to volume with Ultrapure™ Distilled Water (Life Technologies). Additional qPCRs were conducted for filtration, extraction, and PCR controls.

All qPCRs were prepared in dedicated clean room facilities at the TrEnD Laboratory, Curtin University, and amplified with the following PCR cycling conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final extension of 72°C for 10 min. Each sample was amplified in duplicate in a single-step process via the use of fusion-tagged primer architecture that contains a unique 6–8 bp multiplex identifier tag (MID-tag). MID-tagged PCR amplicons were pooled at equimolar ratios based on qPCR ΔRn values and quantified concentrations with the Qubit 3 (Invitrogen; Carlsbad, USA) and QiaExcel (Qiagen; Venlo, Netherlands) instruments. Therefore, each sample was represented in equimolar for sequencing. Three final libraries were constructed and size-selected (160–600 bp) using a Pippin-Prep (Sage Science, Beverly, USA). Size-selected libraries were then purified using the QIAquick PCR Purification Kit (Qiagen, Venlo, Netherlands), quantified using a Qubit 4.0 Fluorometer (Invitrogen, Carlsbad, USA), and diluted to 2 nM for loading onto 500 cycle MiSeq® V2 Standard Flow Cells. Paired-end sequencing was conducted on an

TABLE 1 PCR assay information for benthic eDNA metabarcoding in Lalang-garram marine parks. Both PCR assays target the nuclear ribosomal internal transcribed spacer 2 (ITS2) region

PCR Assay	Target taxa	Primer Name	Oligonucleotide Sequence (5′–3′)	Target Length (bp)	Annealing temp (°C)	Primer reference
CoralITS2	Scleractinia (exempting *Acropora*)	SCLER5.8SForw	GARTCTTTGAACGCAAATGGC	231–437	55	Brian et al. (2019)
		SCLER28SRev	GCTTATTAATATGCTTAAATTCAGCG			
CoralITS2_acro	Scleractinia	SCLER5.8SForw	GARTCTTTGAACGCAAATGGC	220–440	55	Brian et al. (2019)
		CoralAcro_874Rev	TCGCCGTTACTGAGGGAATC			Alexander et al. (2020)

Illumina MiSeq platform (Illumina, San Diego, USA), housed in the TrEnD Laboratory at Curtin University, Western Australia.

2.3 | Bioinformatics

Sequences were demultiplexed into the respective samples based on their MID-tags using the insect package (Wilkinson et al., 2018), and quality filtered (minimum length = 60, maximum expected errors = 2, no ambiguous nucleotides), dereplicated, denoised (pool = TRUE), merged (20bp overlap, no mismatches), and filtered for chimeras using the DADA2 pipeline (Callahan et al., 2016) in RStudio (v1.1.423; R Core Team, 2015). Because of the multicopy nature of ITS2 and variable length of the gene, no strict trimming was performed as recommended by the developers pipeline (Callahan et al., 2016). Resulting amplicon sequence variants (ASVs) for each assay, that is, unique sequences that can be separated by one or more nucleotide differences (Callahan et al., 2017), were then queried against NCBI's GenBank nucleotide database (Benson et al., 2005; accessed in 2020) and the addition of a custom coral ITS2 database (Dugal et al. *under review*, GenBank accession numbers MW473514-MW473666) using BLASTn (minimum percentage identity of 90, maximum target sequences of 10, reward value of 1) via Zeus, an SGI cluster, based at the Pawsey Supercomputing Centre in Kensington, Western Australia. Taxonomic assignments of ASVs were curated using a lowest common ancestor (LCA) approach (https://github.com/mahsa-mousavi/LCA_taxonomyAssignment; Mousavi-Derazmahalleh et al., 2021), which compared the top ten hits for each query and collapsed to the LCA if the percentage identity between each consecutive hit differed by less than one (based on 100% query coverage). In order to compare assignments on both an ASV and taxonomic level (e.g., species and genus), ASVs that shared the exact same taxonomy assignment were merged using the phyloseq "tax_glom" function (McMurdie & Holmes, 2013) in RStudio.

2.4 | Statistics

To investigate the local detectability of sessile benthic eDNA signals from seawater, that is, whether seawater can detect the presence of the locally sampled benthic organisms, we conducted a series of ASV and taxa accumulation analyses. Each benthic tissue sample provided multiple ASVs and subsequent taxa assignments representing the host and also a community of organisms harbored by corals and other sessile benthic invertebrates. Therefore, for each respective site, we produced four accumulation curves by calculating the percent proportion of (1) all tissue ASVs; (2) the top tissue ASVs (i.e., the ASV with the highest read count in each tissue sample and thus presumably the host ASV); (3) all tissue taxa and; and (4) the top tissue taxa (i.e., the taxa with the highest read count in each tissue sample and thus presumably the host taxa) detected in the surrounding seawater, by the addition of sampling replicates at each site.

To examine the resolution of the ITS2 gene region and therefore its ability to accurately distinguish taxa, we calculated the proportion of assignments confidently matched at various taxonomic levels (i.e., species, genus, and family) and investigated the utility of custom reference databases to resolve species-level assignments. We also compared the morphologically identified benthic tissue samples with their corresponding genetic assignments (resulting from both the CoralITS2 and CoralITS2_acro assays). This allowed us to calculate the percent of correct genetic to morphological assignments, tissue samples that did not amplify with the applied primer assays, those that could not be genetically resolved because of inadequate interspecific variation or lack of reference sequences, and tissue samples that were incorrectly assigned based on purported incorrect GenBank reference sequences. The latter was determined by aligning the sequenced ITS2 region from each morphologically identified tissue sample with the respective species reference sequence on GenBank; those that exhibited 5% or more identity variation (with 100% query coverage) were flagged as being potentially incorrect GenBank submissions as a result of misidentification.

The detections of hard corals from eDNA metabarcoding (seawater replicates merged by site) were then compared with the belt transect data at each respective sampling site in the Lalang-garram marine parks. All comparisons were made at a genus level to account for species gaps in the coral ITS2 reference sequence databases. Observed hard coral genus diversity between the eDNA and belt transect approaches was calculated and graphed using ggplot2 (Wickham, 2016) and tested for significance by an ANOVA in RStudio. Environmental DNA (read count) and belt transect (specimen count) abundance data were then standardized to relative composition at each site and converted to a Bray–Curtis similarity matrix in PRIMER v7 (Clarke & Gorley, 2015). Community composition was visualized by nonmetric multidimensional scaling (nMDS) and tested for variation between survey approaches (eDNA versus. belt transect) and between sites using a permutational multivariate analysis of variance (PERMANOVA) in the PERMANOVA + add-on (Anderson et al., 2008) of PRIMER v7. Additionally, these ordination and statistical analyses were also conducted for the presence–absence of eDNA (read count) and belt transect (specimen count) abundance data, which was then converted to a Jaccard similarity matrix in PRIMER v7. To assess whether the abundance of eDNA metabarcoding reads can be used as a proxy for population abundance in the field, we plotted the relative abundance of hard coral eDNA metabarcoding reads against the relative abundance of belt transect hard coral counts at each site and fitted a linear model regression line using the ggplot2 "geom_smooth" function (Wickham, 2016) and "lm" function in RStudio. This was also conducted on the log-transformed relative abundance data for each survey approach. Finally, hard coral coverage (%) was plotted against hard coral genus diversity detected by the eDNA metabarcoding and belt transects at each of the seven sampled sites to examine continuity between the two survey approaches. A linear model was additionally fitted to determine whether the two survey approaches significantly differed in their measure of hard coral genus diversity across the levels of observed hard coral coverage.

3 | RESULTS

3.1 | Local detectability of sessile benthic eDNA

The two eDNA metabarcoding assays yielded a total of 23,482,066 sequencing reads. The mean number of filtered sequences (post-quality, denoising, and chimera filtering) was 63,817 ± 45,311 per tissue and seawater replicate sample for the CoralITS2 assay; and 50,302 ± 32,413 per replicate sample for the CoralITS2_acro assay (Table S2). ASVs that were detected in the filtration, extraction, and PCR controls were flagged as potential cross-contaminants and completely removed from subsequent analyses. This included ASVs that produced detection hits (in low abundance, <50 reads) for the following cnidarian (*Palythoa yoron*), sponge (*Hyrtios erectus* and *Spheciospongia solida*), and hard coral species (*Acropora digitifera*, *A. millepora*, *A. muricata*, *A. robusta*, *Cynarina lacrymalis*, *Cyphastrea japonica*, *Duncanopsammia axifuga*, *Lobophyllia radians*, and *Plesiastrea versipora*).

The ASV accumulation analyses indicated that on average, the CoralITS2 assay detected 28.7% ± 12.6 and the CoralITS2_acro 19.0% ± 15.0 of all tissue ASVs in the surrounding seawater at each respective site (Figure 2a). When examining only the top tissue ASVs (i.e., the most abundant ASV per tissue sample), the CoralITS2 assay detected on average 43.5% ± 34.2 and the CoralITS2_acro 42.4% ± 37.2 (Figure 2b). Detection was also analyzed on a taxonomic level by collapsing ASVs that share the same genetically assigned taxonomy. The taxa accumulation analyses indicated that on average, the CoralITS2 assay detected 71.5% ± 19.4 and the CoralITS2_acro 69.4% ± 14.8 of all tissue taxa in the surrounding seawater at each respective site (Figure 2c). When examining only the top tissue taxa (i.e., the most abundant taxa assignment per tissue sample), the CoralITS2 assay detected on average 68.6% ± 19.3 and the CoralITS2_acro 72.6% ± 20.5 (Figure 2d).

3.2 | Resolution of the Coral ITS2 markers

A total of 214 genetically assigned taxa comprised of 1,817 ASVs were detected in the collected water and tissue samples using the CoralITS2 assay (Figure 3a; Table S3). Predominant families were the hard corals Merulinidae (24 taxa), Acroporidae (20), Lobophylliidae (14), Poritidae (13), and Dendrophylliidae (8). The majority of the

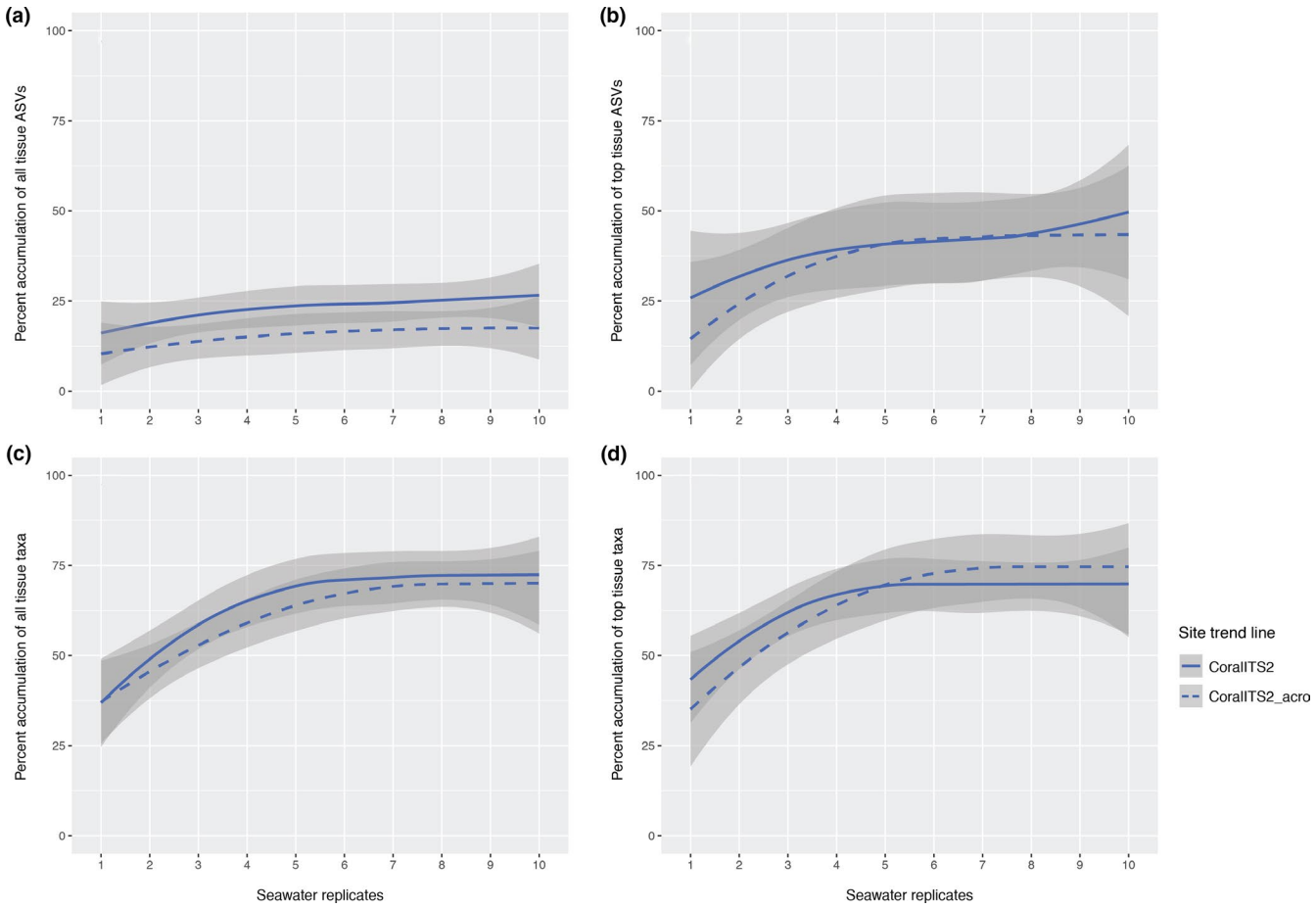

FIGURE 2 ASV accumulation analysis of (a) of all tissue ASVs and (b) top tissue ASVs (i.e., the most abundant ASV per tissue sample), and taxa accumulation analysis of (c) of all tissue taxa and (d) top tissue taxa (i.e., the most abundant taxonomic assignment per tissue sample) in the surrounding seawater, by the addition of seawater replicates, averaged across all sites. Site trend lines are depicted in blue with 95% confidence intervals in gray

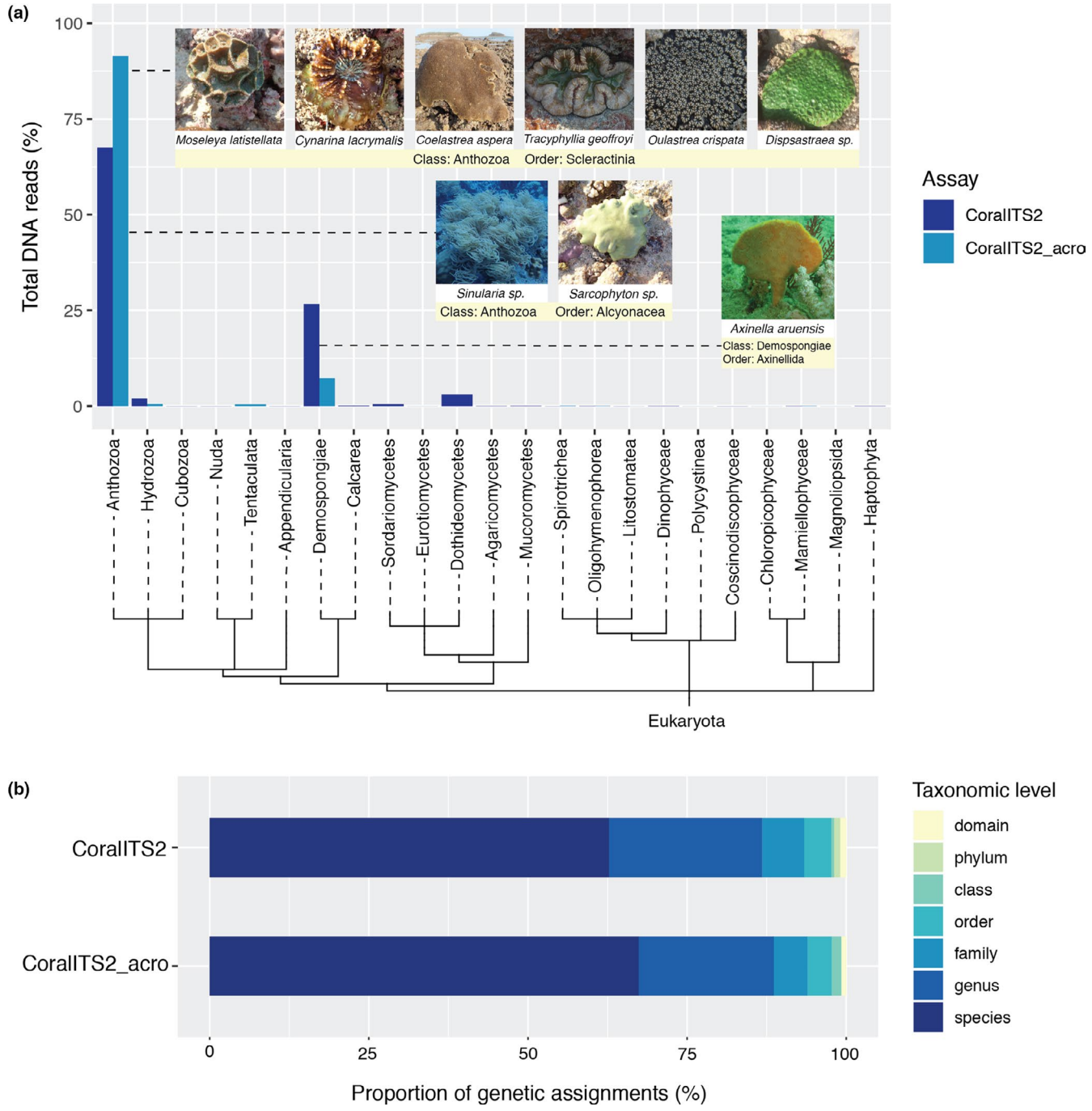

FIGURE 3 (a) Classes detected across water and benthic tissue samples in the Lalang-garram marine parks. The percentage of total DNA reads for each detected class indicates that the CoralITS2 and CoralITS2_acro assays largely target hard corals (Class Anthozoa, Order Scleractinia), soft coral (Class Anthozoa, Order Alcyonacea), and sponges (Class Demospongiae, Order Axinellida). A small selection of benthic specimens genetically detected in this study and photographed onsite are illustrated (Photos by Zoe Richards). (b) Proportion of genetic assignments at various taxonomic levels for the CoralITS2 and CoralITS2_acro detections (both water and benthic tissue samples) in the Lalang-garram Marine Park

detected taxa were assigned (applying the LCA algorithm) at a species level (136), followed by genus only (51), family (14), order (9), class (1), phylum (2), and domain (2) (Figure 3b). These taxonomic assignments are the result of blasting against publicly accessible reference sequences in GenBank (accessed in 2020) and a recently developed coral ITS2 database (Dugal et al. *under review*); the use of the latter resolved six species-level assignments than the use of GenBank on its own. For the CoralITS_acro assay, a total of 136 genetically assigned taxa were detected in the collected water and tissue samples, comprised of 1,386 ASVs (Figure 3a; Table S3). Predominant families were the hard corals Acroporidae (23 taxa), Merulinidae (11), Poritidae (8), and Fungiidae (8). The majority of the detected taxa were assigned (applying the LCA algorithm) at a species level (91), followed by genus only (28), family (7), order (5), class (2), domain (1),

and phylum (0) (Figure 3b). The use of the coral ITS2 database (Dugal et al. *under review*) resolved 10 species-level assignments more than the standard GenBank.

In comparing the morphologically identified benthic tissue samples with their corresponding genetic assignments, we report that only a small proportion were correctly genetically identified (18% and 9% applying the CoralITS2 and CoralITS2_acro assays, respectively; see Figure 4; Table S4). While the CoralITS2 and CoralITS2_acro assays target slightly different coral taxa (the latter optimized for *Acropora*), both assays were unable to amplify 42% of the benthic tissue assemblage collected in this study (Figure 4; Table S4). Through mismatches in the forward and/or reverse primer, the CoralITS2 assay was unable to amplify select hard coral taxa, which included *Acropora* (9 species), *Isopora* (1), and *Caulastraea* (1). The CoralITS2_acro assay was additionally unable to amplify select hard coral taxa; this included *Acropora* (2 species), *Duncanopsammia* (2), *Moseyleya* (1), *Caulastraea* (1), *Alveopora* (1), *Catalaphyllia* (1), *Cynarina* (1), and *Pectinia* (1). Both the CoralITS2 and CoralITS2_acro assays were unable to amplify the soft coral tissues from Octocorallia (1) and *Subergorgia* (1), the sponge tissues from Carteriospongia (1) and Porifera (3, 4, respectively), and lastly tissue from a hydroid (1) and tunicate (1). The remaining genetic assignments were ambiguous, either because there were no ITS2 reference sequences (with 100% query coverage) for the morphologically identified species (25% and 31% applying the CoralITS2 and CoralITS2_acro

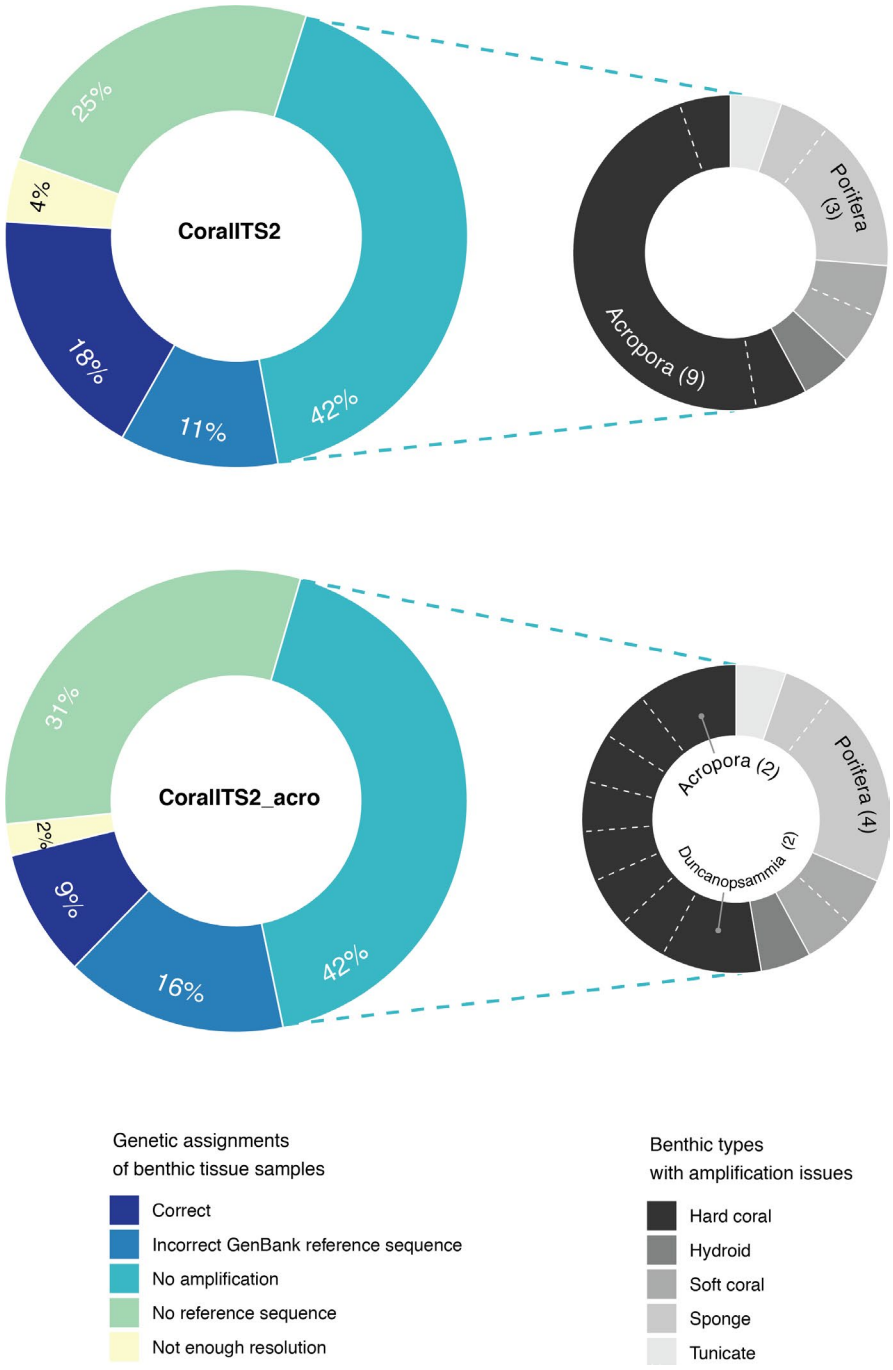

FIGURE 4 Resolution of the genetic assignments attributed to the benthic tissue samples collected in the Lalang-garram marine parks. The smaller donut graphs depict the proportion of benthic taxa that were un-amplifiable with the respective coral metabarcoding assays. Only taxa that represent more than 10% of the total unamplified assemblage were transcribed in these graphs

assays, respectively), there were incorrect GenBank ITS2 reference sequences resulting from misidentification (11% and 16%, respectively), or there was not enough resolution within the ITS2 region to distinguish closely related species (4% and 2%, respectively; see Figure 4; Table S4).

3.3 | Comparison to conventional transect survey

Hard coral genus-level detections were significantly higher using eDNA metabarcoding (combined use of the CoralITS2 and CoralITS2_acro assays produced an average of 39.6 genera ± 14.2 per site) than a belt transect approach (24.1 ± 5.7 per site) across the seven intertidal sites (p <.001; Table S5, Figure 5a). In total, eDNA metabarcoding produced 57 genus-level assignments of hard coral taxa compared with 48 genus-level assignments using a belt transect survey (Figure 5a). Both eDNA metabarcoding and the belt transect survey detected 31 shared genera (Figure 5b), which included hard corals such as *Acropora*, *Montipora*, *Goniopora*, *Porites*, *Platygyra*, *Isopora*, and *Cyphastrea*. The belt transect survey detected 17 genera that were not detected by eDNA metabarcoding (Figure 5b), and this included hard corals such as *Caulastraea*, *Astreopora*, and *Pectinia* (see Table S6 for the belt transect detection list). Likewise, eDNA metabarcoding detected 26 genera that were not detected by the belt transect (Figure 5b), and this included hard corals such as *Favia*, *Cynarina*, *Alveopora*, and *Plesiastrea* (see Table S3 for the complete eDNA metabarcoding detection list). The hard coral community composition detected by the two survey approaches is significantly distinct, as visualized in both the relative abundance and presence–absence nMDS ordinations (Figure 5c and Figure 5d) and confirmed by PERMANOVA (p =.002; Table 2).

The linear regression analysis between the relative abundance of belt transect hard coral counts and relative abundance of eDNA metabarcoding hard coral sequencing reads demonstrated a weak correlation (R^2 = 0.016; Figure 6a; Table S7). A log-linear regression analysis using log-transformed relative abundance data only slightly improved this correlation (R^2 = 0.128; Figure 6b; Table S8). In examining trends in observed diversity between the eDNA metabarcoding and belt transect surveys, a linear regression analysis indicated that the two survey approaches did not significantly differ in their measure of hard coral genus diversity across the levels of observed hard coral coverage (Figure 7; p =.369; Table S9). This signifies continuity between the two survey approaches in detecting patterns of genus diversity across the surveyed sites.

4 | DISCUSSION

4.1 | How locally detectable are sessile benthic eDNA signals?

The application of eDNA metabarcoding for rapid sessile benthic bioassessment holds considerable potential (Alexander et al., 2020; Dugal et al. *under review*; Nichols & Marko, 2019). However, there remain a number of questions regarding the localization of benthic eDNA signals, and the efficiency and accuracy of eDNA metabarcoding as a bioassessment tool compared with conventional benthic surveying techniques. In this study, we firstly investigated the detectability of sessile benthic eDNA signals from seawater by quantifying the eDNA detection rate of locally sampled and sequenced benthic invertebrates. We report that sessile benthic eDNA is not highly (locally) detectable on an ASV (individual/population) level, detecting only approximately 40% of unique ASV signals released by individual local benthic organisms. This relatively low ASV detection rate is at odds with another eDNA seawater haplotype analysis, albeit with whale shark (*Rhincodon typus*), that reported that eDNA detected all haplotypes from collected tissue samples and were at similar frequencies to the population (Sigsgaard et al., 2016). Given our relatively low ASV detection rate, we do not at present recommend using eDNA-derived ASV data for potential population haplotype analyses of sessile benthic taxa, as demonstrated for other taxonomic groups (Aylward et al., 2018; Elbrecht et al., 2018; Parsons et al., 2018; Sigsgaard et al., 2016; Stat et al., 2017).

Even with a limited number of seawater samples per site, eDNA metabarcoding presented a high detection rate of sessile benthic taxa at a taxonomic (e.g., species, genus) level (~70%). Moreover, our accumulation curves suggested that increasing this sample number would not detect significantly more taxa. This rate is comparable to a recent assessment of eDNA metabarcoding and visual surveys (wide and point intercept transects) of coral taxa at the Cocos (Keeling) Islands (Alexander et al., 2020). The applied ITS2 assays detected 58% of the genus assemblage identified visually along the transects. However, it should be noted that both eDNA metabarcoding and the visual surveys also detected several additional genera not detected by the other method (Alexander et al., 2020). A further comparison of the ITS2 assays at the Rowley Shoals, north-western Australia, determined that eDNA metabarcoding only detected 53% of the visually identified coral genus assemblage, although again both approaches detected additional genera (Dugal et al. *under review*).

These relatively high levels of detectability, however, are at odds with a recent eDNA survey that compared molecular operational taxonomic units (MOTUs; equivalent to ASVs) retrieved from benthic scrapes with those from replicate water samples at four distances from rocky-substrate benthic communities (Antich et al., 2020). The authors found that only 7.5% of detected MOTUs were shared between the benthos and water samples; only 5.1% of which were identified as benthic taxa, indicating a very low level of benthic DNA that was locally detectable in the adjacent water. This level of detectability rapidly decreased with distance (up to 20 m) from the benthic source communities (Antich et al., 2020). Another benthic macroinvertebrate assessment, applying short COI metabarcoding of sediment collected along the Basque coast, indicated that metabarcoding was only able to retrieve, on average, 20% of the morphologically identified macroinvertebrate taxa across the sampled sites (Aylagas et al., 2018). The authors note, however, that PCR bias caused by variable primer-template mismatches with the

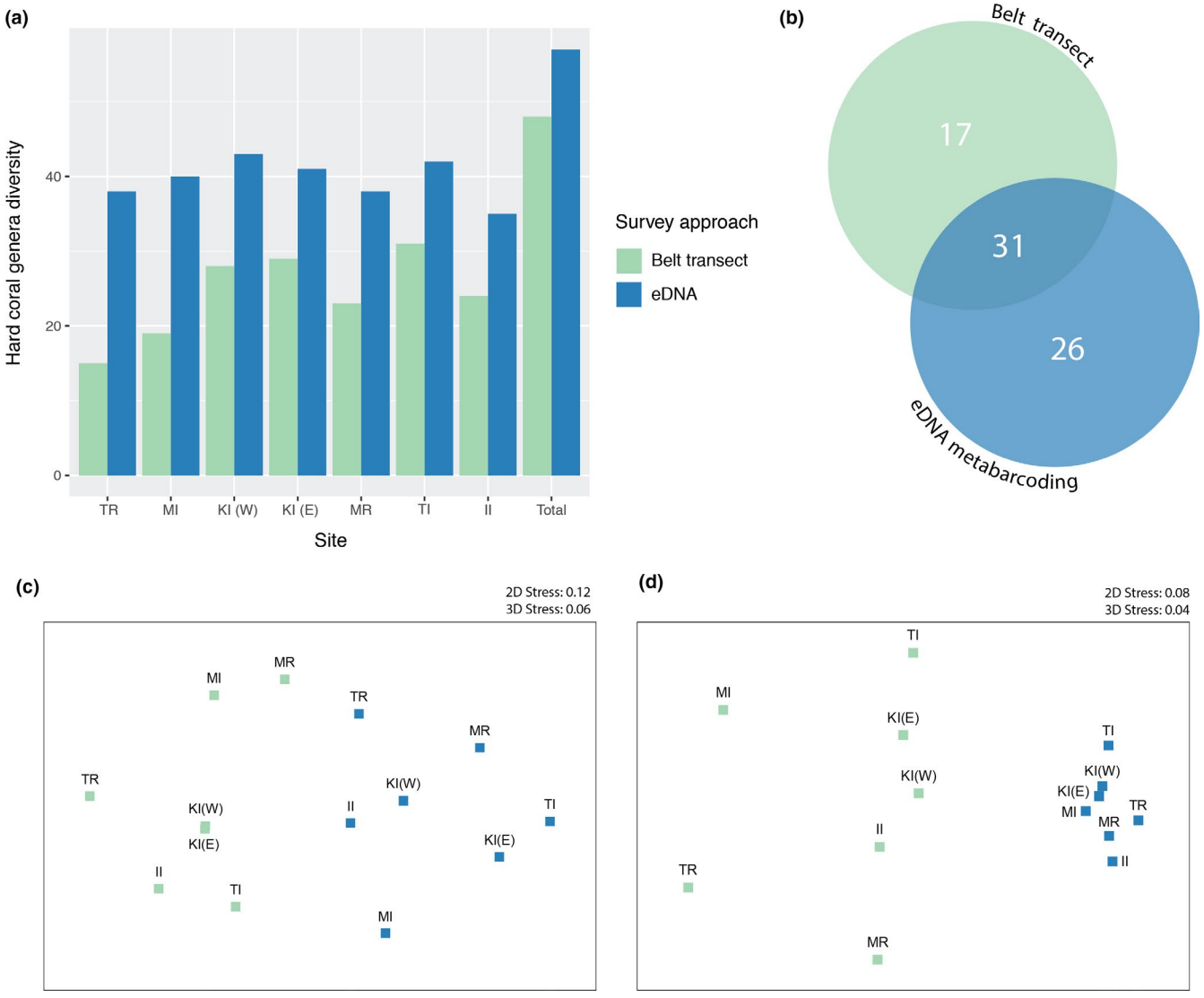

FIGURE 5 (a) Hard coral genus diversity as detected by belt transects and eDNA metabarcoding (CoralITS2 and CoralITS2_acro combined) at each surveyed site and total combined sites. Site abbreviations are as follows: Turtle Reef, TR; Molema Island, MI; Kingfisher Island (Mooloogoob) West, KI (W); Kingfisher Island (Mooloogoob) East, KI (E); Montgomery Reef (Yowjab), MR; Traverse Island Group, TI; and the Iron Islands, II. (b) Venn diagram of the number of unique hard coral genera detected by belt transects and eDNA metabarcoding, and the number of shared hard coral genus detections using these survey approaches. (c) Nonmetric multidimensional scaling (nMDS) ordination plot of relative abundance hard coral genus-level community compositions, color coded by survey approach. Relative abundance of eDNA reads and belt transect counts per site was converted to a Bray–Curtis similarity matrix and plotted in a nMDS ordination space. (d) Nonmetric multidimensional scaling (nMDS) ordination plot of presence–absence hard coral genus-level community compositions, color coded by survey approach. The presence–absence of eDNA reads and belt transect counts per site was converted to a Jaccard similarity matrix and plotted in a nMDS ordination space

applied assay, in addition to variable resolution within the targeted marker, may have compromised the eDNA metabarcoding detection rate in this study.

Unlike the Aylagas et al., (2018) study however, we were able to assess the recovery and local detectability of eDNA signals by isolating the influence of primer biases. This was because we directly applied the same ITS2 assays to both the seawater and collected sessile benthic tissue samples, and we only assessed the presence of successfully amplified benthic tissue taxa in the corresponding seawater samples. Our study indicates that irrespective of PCR assay choice, there is a relatively high detectability rate of eDNA signals in seawater from sessile benthic taxa. However, the selection of a suitable primer assay that minimizes primer biases is critical to maximize the recovery of local benthic eDNA signals. This was demonstrated by the application of a gene enrichment approach (which eliminates PCR bias) that subsequently proved more efficient in the detection of macroinvertebrates in comparison with COI amplicon metabarcoding of the same eDNA samples (Dowle et al., 2016). The contrasting level of local detectability between this study and the Antich et al. (2020) study, however, both of which followed a similar approach in quantifying benthic signals in adjacent water, indicates that at present, we should be cautious in widely applying eDNA

TABLE 2 Permutational multivariate analyses of variance (PERMANOVA) of hard coral genus-level community compositions testing for variation across sites and survey approaches

Data	Source of variation	*df*	Sum of squares	Mean Sq	Pseudo-*F*	*p*
Relative abundance	Site	6	11,943	1990.5	1.2696	.185
	Survey approach	1	10,074	10,074	6.4253	.002**
	Residuals	6	9,407.2	1567.9		
	Total	13	31,424			
Presence–absence	Site	6	6,179.9	1,030	1.3128	.081
	Survey approach	1	8,013	8,013	10.213	.002**
	Residuals	6	4,707.4	784.57		
	Total	13	18,900			

Note: The survey approaches are comprised of belt transects and eDNA metabarcoding of seawater samples at respective sites. A PERMANOVA was conducted for both relative abundance and presence–absence of belt transect (specimen count) and eDNA (read count) hard coral abundance data. Number of permutations: 999. Significant *p* value codes are as follows: 0 < .001 "***", .001 < .01 "**", .01 < .05 "*".

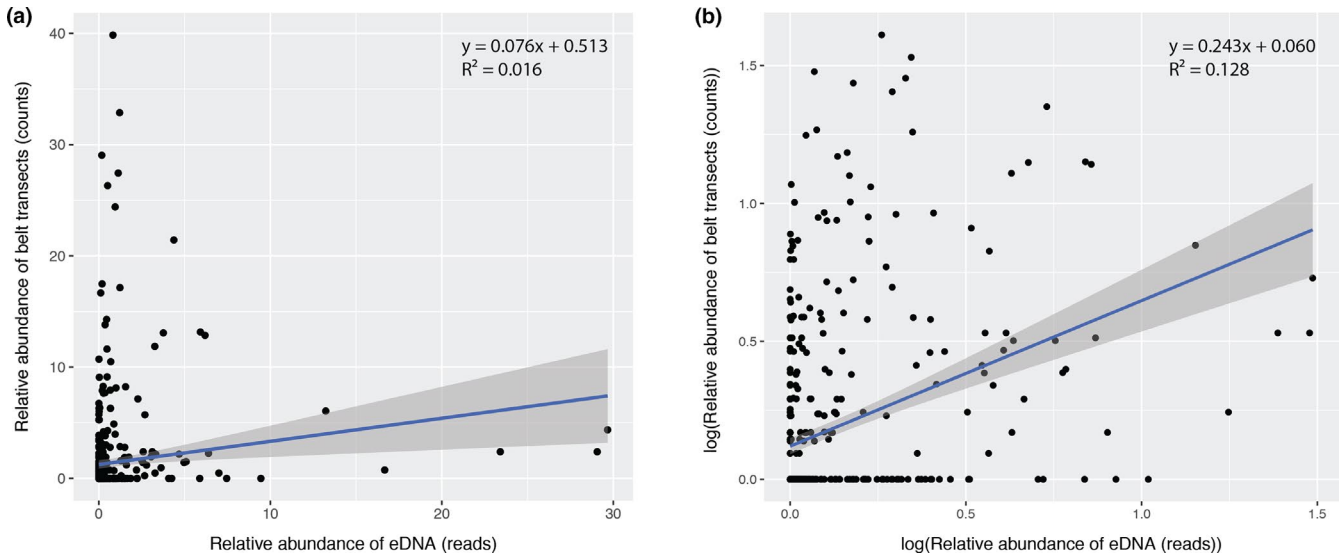

FIGURE 6 Scatterplots illustrating a weak relationship between the (a) relative abundance of hard coral (order: Scleractinia) counts from belt transects with the relative abundance of hard coral sequencing reads from eDNA metabarcoding, and (b) log-transformed relative abundance data. Each black circle represents a genus detection at a site. The fitted linear model is depicted by the blue line with a 95% confidence interval in gray. The fitted linear model function and R-squared value are provided in the top right-hand corners, respectively. Regression statistics are provided in Tables S7 and S8

metabarcoding as a stand-alone technique for benthic surveying. Further comparative research with conventional benthic surveying in various environments is required.

4.2 | What level of resolution can (at present) be gleaned from eDNA approaches?

The application of the ITS2 assays to collected seawater samples in the Lalang-garram marine parks produced over 200 taxonomic assignments—the majority of which were assigned at a species level using an LCA algorithm (Mousavi-Derazmahalleh et al., 2021). Sixteen of these assignments were resolved to a species level by blasting against a locally curated database for Western Australian corals (Dugal et al. *under review*). This highlights the importance of barcoding regional specimens for reference databases. However, not all of these species-level assignments had a high percentage identity match (i.e., over 98%), but were accepted at a species level because there were no other hits within a close percentage match (i.e., at or within 1% difference) that additionally satisfied the criteria of 100% query coverage. This indicates that despite a high level of observed resolution within the ITS2 markers, the resulting genetic assignments/community compositions could be further refined with additional reference databases for ITS2.

To examine the accuracy of the applied ITS2 assays for future bioassessment surveys, we directly compared the resulting

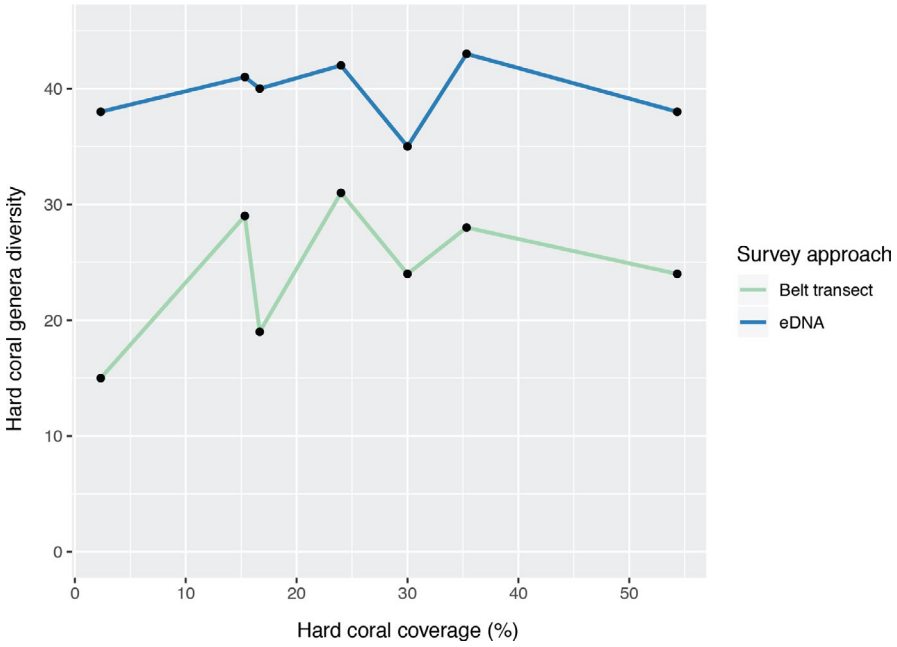

FIGURE 7 Line plot depicting the relationship between hard coral genus detection and hard coral coverage (%) at the surveyed sites (black circles) in Lalang-garram marine parks. The belt transects and eDNA metabarcoding approaches show continuity in hard coral genus detection across varying levels of hard coral coverage, the latter obtained in the point intercept transects

metabarcoding and morphological assignments of the collected sessile benthic tissue samples. This analysis revealed several issues in correctly assigning taxonomies and characterizing sessile benthic community composition using the applied ITS2 assays. A large proportion (42%) of the benthic tissue sample assemblage could not be directly amplified. This included some hard corals (such as *Caulastraea* sp. and *Acropora millepora)*, but largely compromised soft corals (such as *Subergorgia* sp. and Octocorallia), sponges (such as *Carteriospongia foliascens*), a tunicate, and hydroid. Therefore, it may be beneficial to create a separate assay optimized for soft coral, sponge, tunicate, and hydroids. Moreover, up to 31% of the genetic identifications of the benthic tissue sample assemblage had no ITS2 reference sequences (with 100% query coverage) for morphologically identified species. At present, there are still large gaps in ITS2 reference material for sessile benthic data on publicly accessible databases, such as NCBI's GenBank. Furthermore, many of these submitted ITS2 sequences on GenBank do not completely cover the ITS2 gene region and are thus redundant when applying 100% query coverage with these assays. We therefore highly recommend extending ITS2 databases for greater regional representation, in this case north-western Australian corals. Until this is adequately addressed, it may be more appropriate to collapse species assignments to a genus level, as implemented in our comparison to the conventional transect survey.

Another issue that broadly plagues all eDNA metabarcoding research is the considerable number of unidentified, misidentified, and outdated synonyms of specimens on GenBank (Strasser, 2008). In this study, we identified that up to 16% of the benthic tissue samples were incorrectly assigned based on purported incorrect GenBank reference sequences. Unfortunately, with the potential implementation of eDNA metabarcoding as a stand-alone monitoring technique it will become incredibly difficult to flag incorrect GenBank sequences. To minimize the effect of incorrect GenBank sequences, we suggest ignoring taxonomic hit outliers, for example, if 9/10 BLAST hits are for the same species, and 1/10 is for another species, then we suggest ignoring the latter. However, we recognize that this does induce selection bias. To completely circumvent the issue of unidentified or misidentified specimens on GenBank, we highly recommend using curated reference databases, preferable local, which will additionally help to ensure that phylogeographic variation is accounted for.

Lastly, the multicopy nature of the internal transcribed spacers (ITS1 and ITS2) may present issues in correctly assigning benthic taxonomies using targeted ITS assays. These ITS regions act as regulators, situated between nuclear ribosomal DNA (rDNA) genes that encode ribosomal RNA subunits across all eukaryotic genomes. The rDNA genes and associated spacers are arranged in one of more large arrays that occur in tandem repeats between hundreds and thousands of copies long. These multicopy genes typically evolve simultaneously through "concerted evolution" and are homogenized through recombinant processes, such as unequal crossover and gene conversion (Elder & Turner, 1995). However, they can vary from introgression via hybridization and evolution at different chromosomal positions (Hillis & Dixon, 1991; Muir et al., 2001). Notably, the high rate of mutation across these arrays has promoted the use of ITS1 and ITS2 above other mitochondrial markers for species-level discrimination in scleractinian corals (Chen et al., 2004; Marquez et al., 2003). In phylogenetic reconstructions, multicopy variation is largely dealt with by only analyzing the consensus ITS sequence from a single-source sample (Wang et al., 2017). It is anticipated, however, that an environmental sample will contain a myriad of these copies, which may be difficult to delineate whether they represent intragenomic variability (i.e., multiple ITS sequences from the same individual), intrapopulation variation (between individuals from the same colony), and interpopulation variation (between colonies). Importantly,

significant variation may affect robust taxonomic assignments of multicopy ITS sequences to a reference sequence.

Pairwise comparisons of ITS intragenomic variability were found to differ on average by 0.56% ± 0.49% in a study of scleractinian corals (Forsman et al., 2006). This was lower than intrapopulation variation (0.95% ± 0.51%), interpopulation variation (1.18% ± 0.45%), and lastly, interspecific variation (22.55% ± 1.99). Based on these estimates which indicate that intraspecies variation is within 2%, we do not anticipate that the multicopy nature of the ITS regions will adversely impact species-level taxonomic assignments (typically ≥ 98%) from metabarcoding data. The multicopy nature of the ITS regions will, however, lead to the amplification of multiple ASVs from the same individual, confounding estimates of population variability. As previously discussed, we do not recommend using eDNA-derived ASV data for potential population haplotype analyses. Ultimately, ITS intragenomic variability, amidst other intra- and interpopulation variation, highlights the need for greater regional representation in databases to achieve robust taxonomic assignments from eDNA metabarcoding.

4.3 | How does eDNA compare to the conventional transect survey?

While the local detectability analyses indicated that we can only reliably glean ~70% of the sessile benthic taxa diversity at a locality with eDNA, the applied ITS2 markers on the collected water samples detected slightly more hard coral genera than that of the belt transects in this study. Nonetheless, both survey approaches detected numerous unique genera and have significantly different community compositions as visualized in the ordination plots. This suggests that a combined approach would provide a higher level of biodiversity coverage for future bioassessments, as previously advocated for corals (Alexander et al. 2020; Dugal et al. *under review*) and other taxonomic groups (Kelly et al., 2017; Port et al., 2016; Stat et al., 2019).

A current limitation of eDNA metabarcoding in comparison with conventional transect surveys is the inability to reliably provide quantitative data, such as population abundance or biomass/coverage. There are a number of eDNA studies that have examined the use of raw sequencing reads or relative proportion of sequencing reads as a proxy for abundance and/or biomass/coverage (Atkinson et al., 2018; Turner et al., 2014; Ushio et al., 2018). However, the majority of these quantification studies have focused on individual species using species-specific primers as eDNA shedding rates vary between both species and taxonomic groups (Sassoubre et al., 2016). The use of multispecies primers for eDNA metabarcoding applications, however, may compromise quantification estimates. Firstly, it is incredibly difficult to incorporate various species shedding rates, particularly when examining a large community composition; secondly, the introduction of PCR bias, whereby DNA from a species that presents perfect homology (or no mismatches) to the primers is preferentially amplified over another species that presents mismatches (Schloss et al., 2011), will subsequently skew downstream read abundance data. PCR bias can only be circumvented if all target taxa present the same homology to the primers, which may be challenging to achieve.

In this study, we assessed the potential quantification of hard coral abundance, by fitting a linear model regression between the relative abundance of hard coral ITS2 reads and relative abundance of belt transect hard coral counts at each site. This was also conducted for log-transformed relative abundance data. Both approaches, however, demonstrated only a weak correlation, indicating that the use of relative abundance reads (as obtained from the ITS2 assays) is unsuitable as a proxy for hard coral counts. This is in contrast to a recent eDNA coral cover survey at Oahu, Hawaii, that demonstrated that visual estimates of log-transformed hard coral coverage highly correlated with log-transformed coral sequencing reads of 16S rDNA and COI (Nichols & Marko, 2019). This discrepancy could potentially be attributed to a higher diversity and thus complexity of hard coral eDNA signals at the Lalang-garram marine parks compared with the sampled sites in Hawaii; the latter study only detecting up to six genera compared with 57 hard coral genera in this study. Given the results of this study, we cannot at present recommend the use of relative abundance sequencing reads as a proxy for hard coral abundance.

Nonetheless, we did detect continuity between eDNA metabarcoding and the belt transect surveys in detecting trends in genus diversity across varying levels of hard coral coverage at the surveyed sites. This indicates that eDNA metabarcoding can distinguish diversity trends between sites as a conventional technique would. This further validates the application of eDNA metabarcoding either as a stand-alone or complementary monitoring technique for hard corals, and more broadly, sessile benthic organisms.

5 | CONCLUSION

This study adds to a growing body of evidence that suggests eDNA metabarcoding is an auspicious approach for undertaking rapid sessile benthic bioassessments, and it holds considerable potential for application in remote, turbid, and dangerous locations such as the inshore Kimberley. This study also provides the foundation from which a regional eDNA-based benthic monitoring program can be developed. At present, reporting at the level of genera is recommended; however, the likelihood of enhancing the accuracy of species-level assignments is expected to grow in tandem with an increase in local reference sequences. Combining eDNA metabarcoding with other survey techniques is the most effective way to provide a more holistic representation of benthic biodiversity, and we anticipate such combined approaches will greatly increase the scope of benthic monitoring in complex ecosystems such as the inshore Kimberley.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

ZTR, KW, and AA conceived the study; ZR secured project funding; WR, DB, AL, ZR, and AA fieldwork logistics; AA, ZR, WR, DB, and AL participated in fieldwork; AA contributed to laboratory work, KW contributed to statistical analysis; KW, ZR, and AA wrote the manuscript; all authors edited the manuscript.

DATA AVAILABILITY STATEMENT

The ITS2 sequencing data have been uploaded to GenBank accession numbers MW473514-MW473666. Demultiplexed (unfiltered) fastq sequencing data and ASV/taxonomic (read abundance) matrices for both the ITS2 and ITS2_acro assays are available for download on Dryad Digital Repository (https://doi.org/10.5061/dryad.xksn02vdx).

[Correction added on 1 March 2021, after first online publication: Data Availability Statement has been modified.]

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SUPPORTING INFORMATION

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

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