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Multispecies environmental DNA metabarcoding sheds light on annual coral spawning events

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Abstract

Synchronous multispecific coral spawning generally occurs annually and forms an integral part of the coral life cycle. Apart from spawning times and species participation, however, much else remains unknown. Here, we applied environmental DNA (eDNA) metabarcoding to study two tropical reef sites of contrasting coral cover before, during and after coral spawning. Using coral-ITS2 and vertebrate-12S markers, we evaluated eDNA as an alternative monitoring tool by assessing its capabilities in detecting spawning species and tracking relative abundances of coral and fish eDNA. Over 3 years, elevated eDNA coral signals during the event (proportional read increase of up to five-fold) were observed, detecting a total of 38 coral and 133 fish species with all but one of the coral species visually observed to be spawning. This is also the first demonstration that eDNA metabarcoding can be used to infer the diurnal partitioning of night- and day-time spawning, spawning in coral species overlooked by visual surveys, and the associated changes in fish trophic structures as an indicator of spawning events. Our study paves the way for applied quantitative eDNA metabarcoding approaches to better study ephemeral and important biological events.

KEYWORDS
Actinopterygii, high-throughput sequencing, Scleractinia, Southeast Asia, synchronous spawning, tropical reefs, water sampling

1 | INTRODUCTION

Coral mass spawning is an ephemeral ecological event typically occurring annually, at night, during specific moon phases (Foster et al., 2018; Levitan et al., 2004, 2011; Lin & Nozawa, 2017; Shlesinger & Loya, 2019a). It is an integral part of the coral life cycle and involves the synchronous release of sperm and eggs by multiple coral species into the water column (Atkinson & Atkinson, 1992; Babcock et al., 1986; Baird et al., 2009; Carlon, 1999). First described in detail four decades ago (Harrison et al., 1984; Shlesinger & Loya, 1985), it is regarded as the planet's largest sexual reproduction event in the marine realm. More importantly, coral spawning is ecologically significant as it directly embodies the future of reef ecosystems (Harrison, 2011; Levitan et al., 2014) and provides information on reef health (Baird, Guest, et al., 2021). Thus, identifying spawning cues and times are essential for effective reef management and conservation strategies (Danylchuk et al., 2011; Gilmour et al., 2016; Spear et al., 2015).

Temperature (Keith et al., 2016), solar insolation (van Woesik et al., 2006), sunset and moonrise timings (Knowlton et al., 1997; Levitan et al., 2011; Lin et al., 2021), tide (Babcock et al., 1986), wind (van Woesik, 2010), precipitation (van Woesik, 2010) as well as lunar cycles (Brady et al., 2016; Foster et al., 2018; Lin & Nozawa, 2017) are proximate cues that can be used to predict the spawning months or even dates for a number of target species. However, we still do not fully understand (i) the links between biotic and/or abiotic cues to the partitioning of spawning times and days among species (Baird, Guest, et al., 2021; Shlesinger & Loya, 1985; van Woesik, 2010), (ii) what is responsible for unusual patterns such as daytime spawning (Bronstein & Loya, 2011; Liu et al., 2005; Mangubhai et al., 2007) and (iii) unsynchronized or split spawning events (Fogarty & Marhaver, 2019; Foster et al., 2018; Hock et al., 2019; Mangubhai & Harrison, 2008; Shlesinger & Loya, 2019a). In situ studies typically rely on visual surveys to document coral spawning, where expert teams trained in coral identification use SCUBA diving and underwater photography to document spawning colonies and identify participating coral species. These visual survey techniques are labour-intensive and susceptible to observer biases, site accessibility and low visibility at night (Miller et al., 2012; Takeuchi et al., 2019). Moreover, the duration of visual surveys is also limited by the observers' capacity to observe spawning events over sufficiently long time frames, on the scale of hours per night or nights per month (Levitan et al., 2004). Critically, surveys that require direct observations may be invasive because of disturbances caused by physical presence of divers in the water (Heyman et al., 2010; Tsuji & Shibata, 2021), which can impact the external fertilization rates among broadcasting corals and alter predatory behaviours of planktivorous fish (Baird et al., 2009; Giglio et al., 2022; Westneat & Resing, 1988). Nevertheless, surveys are needed globally and annually to monitor spawning but due to the rarity and fleetingness of the event, only a limited proportion of the necessary information relating to spawning participation, timing and intensity is available (Shlesinger & Loya, 2019a; van Woesik, 2010). This necessitates development of noninvasive and efficient alternative monitoring tools that would bypass limitations of visual surveys to better study this extraordinary phenomenon.

Over the last three decades, there have been a few attempts at establishing alternative methods to noninvasively monitor coral spawning, for instance through seawater phosphate sampling (Atkinson & Atkinson, 1992) and remote sensing (Jones et al., 2006; Yamano et al., 2020). These techniques have effectively detected spawning events but lack fine-scale resolution on which reef species participate. Environmental DNA (eDNA) metabarcoding holds promise as a noninvasive method with species-level resolution that is suitable for more effective biomonitoring (Shelton et al., 2016; West et al., 2021). The application of marine eDNA methods typically requires collection of seawater that comprises heterogeneous soups of genetic material shed from species across trophic levels (Bayer et al., 2019). In the case of the mass release of gametes during spawning, the eggs and sperm would be the primary sources of eDNA (Bylemans et al., 2017), and concentrating eDNA from seawater allows for collection of valuable ecological information (Bayer et al., 2019; Erickson et al., 2016; Tillotson et al., 2018). Increasingly, analysing eDNA concentrations has drawn meaningful conclusions beyond characterizing species presence (Evans et al., 2016; Everts et al., 2021; Tillotson et al., 2018; Yates, Glaser, et al., 2021). For instance, some studies have demonstrated that an increase in eDNA concentration is contingent on either a sudden surge in species densities or production fluxes in response to environmental and biological stimuli (Bracken et al., 2019; Tillotson et al., 2018). As such, we hypothesize that when compared against a baseline of water samples from

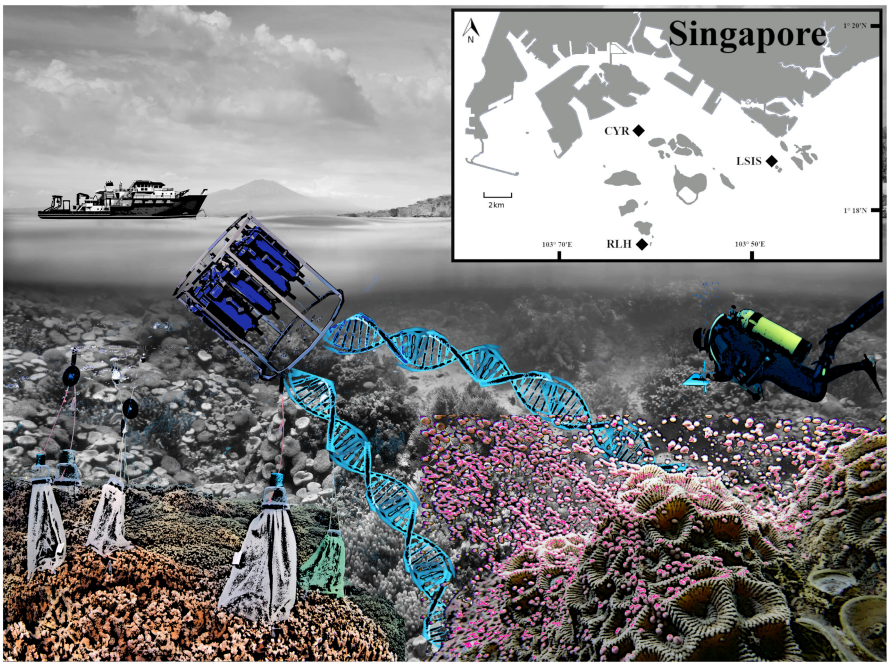

FIGURE 1 Illustration of various methods that can be used to monitor coral spawning, such as visual survey with SCUBA (far right), collection cups and nets placed over spawning colonies for direct sampling of coral gametes (bottom left), and sampling seawater for environmental DNA (eDNA, middle). eDNA is potentially a less invasive approach for studying coral spawning events. The inset map (upper right) shows the three eDNA water sampling sites in Singapore. Cyrene Reefs (CYR) represents a low coral cover site, while Little Sister's Island (LSIS; 2017) and Raffles Lighthouse (RLH; 2018, 2019) represent high coral cover sites. Visual coral spawning surveys were conducted for all years (2017, 2018, 2019) at RLH only.

outside the spawning event (background window), we should detect a spike in coral eDNA relative abundances stemming from the synchronous mass release of gametes during the coral spawning event (spawning window). In addition, feeding frenzies among reef fish constitute another fascinating phenomenon associated with coral spawning (Pratchett et al., 2001; Westneat & Resing, 1988). Since increased levels of predation can provide some indication of the onset of spawning events (Heenan et al., 2020; Westneat & Resing, 1988), we expect that there will also be temporal changes in fish eDNA abundances and potential trophic structure shifts, providing further evidence for coral spawning occurrences.

Here, we tested if multispecies eDNA metabarcoding was able to detect coral spawning events and to identify the participating coral and fish species (Figure 1). Water samples from the tropical reefs of Singapore were collected both at night and day during the spawning window, and the coral-specific nuclear internal transcribed spacer (ITS2) (Flot et al., 2008; Takabayashi et al., 1998) and vertebrate-specific mitochondrial 12S rRNA (Riaz et al., 2011) were targeted in metabarcoding assays to detect spawning corals and active fish species respectively (Pratchett et al., 2001). Visual surveys were also performed to document spawning species and activity for validating the eDNA results, while a comparison of coral egg sizes between species records from both methods was used to evaluate eDNA's detection sensitivity for other spawning species that could have been overlooked due to observer biases. We applied the same approach to sites with both high and low coral cover—Raffles Lighthouse (RLH) or Little Sister's Island (LSIS), and Cyrene Reefs (CYR), respectively (Wong et al., 2018)—to test eDNA's broad utility to biomonitor coral spawning across different reef conditions. More importantly, we explored eDNA's feasibility as a quantitative survey approach for tracking changes in relative abundances of coral and fish species to improve monitoring efficacy of spawning events. We are confident that our findings will shape future monitoring of coral spawning events, given that the techniques are noninvasive, robust and labour-efficient while providing biologically meaningful information associated with this transient event.

2 | MATERIALS AND METHODS

2.1 | Study site selection, visual surveys and eDNA water sampling

Two study sites of contrasting coral cover were selected with the aim of investigating whether eDNA was broadly applicable across reef sites of varying coral abundances (Figure 1). We selected Cyrene Reefs (CYR) as our low coral cover site and Raffles Lighthouse (RLH) as our high coral cover site in 2018 and 2019 based on past survey data (Wong et al., 2018). Briefly, these data were consolidated from benthic line intercept transect (LIT) surveys conducted across 25 coral reefs around Singapore (Wong et al., 2018). Due to logistical constraints encountered in 2017, we substituted RLH with another similar high cover site—Little Sister's Island (LSIS). We subsequently compared 2018 and 2019's eDNA results with those of visual surveys conducted during coral spawning.

Visual diving surveys were carried out at RLH to record participating coral spawning species over 3 years, during the predicted coral spawning window ~3–5 days after the full moon in April (Guest et al., 2002). A fortnight before the predicted spawning window, in situ observations were made to assess spawning likelihood based on presence of maturing eggs in the mouths around the calicle polyps. Several small coral fragments were sampled from colonies ≥20 cm in diameter and visually assessed for egg presence and maturity—specifically, pigmentation intensity to determine readiness of release during the impending spawning event (Guest et al., 2005a; Harrison & Wallace, 1990). A total of 28 colonies across 16 species in 2017, 84 colonies from 29 species in 2018, and 46 colonies from 23 species in 2019 were forecasted to spawn and tagged with plastic labels during prespawning surveys to facilitate follow-up observations during the spawning period (Data S1). During the predicted coral spawning window, spawning visual surveys started as early as 4 p.m. and ended before midnight. For each night, a total of three SCUBA dives, lasting not more than 2 hr each, were conducted by up to 10 observers working in pairs. Tagged colonies that were forecasted to spawn based on assessments from a fortnight prior were closely monitored for timed release of gametes. Confirmatory photographs of the spawning species were taken, along with the documentation of spawning time, species identity and mode of gamete release, that is bundles or separately (Data S1).

Water samples were collected from the above-mentioned reef sites during the coral spawning window between March and April from 2017 to 2019 (Data S1). Although a second spawning window was previously reported to take place between September and October (Guest et al., 2005b), we did not collect water samples during this time as there were no visual survey records for comparison and validation. We performed water sampling 1 week before the spawning window for all years, and in 2019, we sampled 1 week after the event to establish baselines. This facilitated the comparison of eDNA signals between samples collected in the spawning and background window, with the aim of detecting spikes in eDNA relative abundance during spawning. A total of 44 2-L water samples were collected from the subtidal areas at two depths, 1 m (shallow) and 10 m (deep) from the water surface. All water samples were collected using a 5-L Van Dorn water sampler under clear weather conditions. We initially collected water samples only in the day for 2017 and 2018, and later expanded our collection to both day and night water sampling in 2019 to compare detection results with daytime samples (Data S1). Upon collection, water samples were immediately placed on ice and transported back to the laboratory for vacuum-filtering through sterile nylon filter membranes (47 mm diameter; 0.22 μm pore size; Thermo Scientific), which were then stored at −80°C. The time from collection to storage took <3 h.

We applied stringent contamination control measures that included cleaning of all working surfaces onboard the research vessel, laboratory apparatus and sampling equipment with 20% household bleach diluted with Milli-Q water. All collection bottles and filtration

equipment (filter units and membranes) were autoclaved and subjected to UV irradiation in a biological safety cabinet for >30 min. Disposable gloves were also disinfected with 20% bleach prior to use. Post-filtration work was performed in a biological safety cabinet. Negative controls for field collection, DNA extraction and polymerase chain reaction (PCR) were set up and processed in the same way as the samples to identify potential contamination; we used molecular-grade water in place of template DNA for the negative controls.

2.2 | eDNA extraction, metabarcoding and library preparation

Filter membranes were first incubated in 900 µl CTAB (hexadecyltrimethylammonium bromide) with 20 µl of 20 mg ml^{-1} proteinase K for 3 hr at 55°C. The digest was then purified via phase separation with phenol/chloroform/isoamyl-alcohol (25:24:1) and incubated in 60% isopropanol for 16 h at −30°C to increase DNA recovery and yield. DNA pellets were then resuspended in 40 µl of molecular-grade water.

Two gene fragments were targeted for the metabarcoding assay—the 5.8S-internal transcribed spacer 2-28S (ITS2) and 12S-V5 ribosomal RNA (12S). We used scleractinian-specific primers: ITSc2-5 Forward: 5′-AGCCAGCTGCGATAAGTAGTG-3′ (Flot et al., 2008) and ITS-4 Reverse: 5′-TCCTCCGCTTATTGATATGC-3′ (Flot et al., 2008; Takabayashi et al., 1998) to amplify a 300–400-bp fragment. To target fish species, we used vertebrate-specific *ecoPrimers* primer set, Forward: 5′-ACTGGGATTAGATACCCC-3′, and Reverse: 5′-TAGAACAGGCTCCTCTAG-3′ (Riaz et al., 2011) to amplify an 85–117-bp fragment of the 12S locus. Both ITS2 and 12S PCRs were tagged with unique 8-bp barcodes on the 5′ end to allow sequence-to-sample association in the downstream demultiplexing step (Meier et al., 2016). We ensured that each reaction had its own unique sequence tag combination (for up to 96 unique tag combinations for each gene).

Five PCR replicates were performed for each gene per water sample, for a total of 440 reactions (44 samples × 5 replicates × 2 genes). Each PCR mix, comprising a total volume of 25 µl, contained 0.5 µM of each primer (Integrated DNA Technologies), 0.5 µg bovine serum albumin (New England Biolabs), 25 mM magnesium chloride (New England Biolabs), 5 µl template DNA, 9.25 µl sterile water with 1 U BioReady rTaq DNA polymerase with 1× reaction buffer (v/v) (Bulldog Bio). The thermal cycling profile for ITS2 had an initial denaturing step of 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 53°C and 75 s at 72°C, and a final elongation step of 5 min at 72°C; while for 12S it was 95°C for 7 min, 36 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 40 s and a final extension for 5 min at 72°C. PCR amplification success was verified on 2% agarose gels stained with GelRed (Biotium).

A total of 440 tagged amplicon samples and 540 negative controls were combined into 14 pools (up to 96 unique PCRs each; see above) and cleaned using 0.9–1.0× and 1.5–1.8× AMPure XP (Beckman Coulter) for ITS2 and 12S libraries respectively. PCR-free libraries were prepared using NEBNext Ultra II DNA Library Prep Kit (New England Biolabs) following the manufacturer's protocol up to the adapter ligation step (i.e., no PCR enrichment). Each of these 14 library pools was further multiplexed with unique Illumina adapters (Set B), before pooling in equimolar ratios and outsourced for sequencing at the Genome Institute of Singapore. The ITS2 libraries were sequenced over four lanes of Illumina HiSeq 2500 (250-bp paired-end) occupying 20–60% of each lane, while the 12S libraries were sequenced over one full lane of Illumina HiSeq 4000 (150-bp paired-end) and one partial lane of HiSeq 2500. All six sequencing lanes were each spiked with 20–35% PhiX to improve base diversity. Sequencing reads were deposited at the NCBI Sequence Read Archive under BioProject PRJNA801064.

2.3 | Filtering and quality control of Illumina reads

Quality of raw reads was first visualized using FASTQC version 0.11.4. Paired-end reads were then merged using PEAR version 0.9.10 (Zhang et al., 2014). PEAR for ITS2 sequences used the following parameters: minimum assembly length (n) = 250, maximum assembly length (m) = 400 and quality score threshold (q) = 30, while 12S sequences used: minimum assembly length (n) = 80, maximum assembly length (m) = 200 and quality score threshold (q) = 20. OBITOOLS version 1.2.11 (Boyer et al., 2016) was used for demultiplexing and further downstream processing of assembled reads. Using *ngsfilter*, we demultiplexed sequence reads to respective PCR replicates using only the unique forward primer tag and default settings, where no mismatch was allowed for barcode tags, while up to two mismatches were allowed for the primer sequences. Following this, CUTADAPT version 1.18 (Martin, 2011) was used to remove the reverse primer and tag sequences. All successfully demultiplexed and primer-free reads were concatenated into a single file, sequence and records were grouped and dereplicated using *obiuniq*. Finally, sequences were binned into PCR replicate files using *obisubset*.

The data set was filtered for metazoan sequences using BLASTN implemented on BLAST+ version 2.8.1 (Camacho et al., 2009) to match against the NCBI *nt* database (downloaded September 2, 2019), retaining only sequence reads with ≥80% sequence similarity. The output was parsed with READSIDENTIFIER version 1.0 (Srivathsan et al., 2015) to obtain preliminary taxonomic identities of each sequence. This step also allowed us to eliminate nontarget reads from the data set, while allowing a glimpse of other taxa from nonspecific amplification. For the ITS2 marker, the discarded nonmetazoan sequences were normalized within each sample to evaluate the proportion of nonspecific amplification across samples. Quality filters were applied to eliminate reads with amplification and sequencing errors, while ensuring that read coverage was comparable across samples. Next, we used the *obistat* module to summarize the total read count per replicate file, followed by implementing a read count filter for each PCR replicate based on a relative

threshold. Only sequences whose abundance exceeded 0.0001 ($1e^{-4}$) of the total read count for the PCR replicate were used in the analyses (Ip, Tay, et al., 2021). However, we found singleton reads in some replicate files that had met the threshold and these were subsequently excluded by setting a minimum read count of 2. This read count filter was implemented with *obigrep*, while simultaneously retaining sequences of lengths 250–400 and 90–140 bp for ITS2 and 12S respectively. We then used *obiclean* to collapse sequences with potential PCR sequencing errors into respective unique sequence reads. From here, sequences were subjected to another round of BLASTN and READSIDENTIFIER and we retained sequences assigned to Anthozoa with ITS2 and Actinopterygii with 12S samples (≥80% sequence similarity) for further ecological analyses. The non-Anthozoa and non-Actinopterygii reads were also normalized within samples and evaluated to determine proportions of nontarget taxa in the former, and presence of large-bodied vertebrates in the latter that may also be associated with coral spawning events. The non-Actinopterygii sequences from Chondrichthyes, Delphinidae, Sirenia and Testudines were subsequently retained for downstream analyses with Actinopterygii. Sequences in the negative PCR controls were found to be mostly human or chicken in origin (Figure S1). We checked the header names of these negative PCR sequences against sequences from sample PCR replicates, and all matching sequence headers in sample replicates were removed before downstream analyses. Notably, none of the sequences in negative PCRs were of Anthozoa, Actinopterygii, Chondrichthyes, Delphinidae, Sirenia or Testudines origin.

Taxonomic assignment was performed only at the species level, by applying respective delimitation thresholds—species-level identity: ≥97% for ITS2 (Afiq-Rosli et al., 2019) and ≥98.3% for 12S (Sigsgaard et al., 2020)—for each gene (Data S1) to collapse the unique sequence reads into molecular operational taxonomic units (MOTUs) (Škaloud et al., 2018). We eliminated potential false positives by removing MOTUs present in only one PCR replicate and/or taxa that have documented ranges outside the Indo-Pacific. For sequences with higher-level matches to records that were out of the geographical range, we screened the top 10 BLAST matches and selected the next lower percentage matches that were within the species-level threshold and expected geographical range. Additionally, MOTUs that were (i) not assigned species-level identities, or (ii) matched at high percentages (99–100%) to multiple taxa within the same or different genera respectively were also removed from this group of species-level MOTUs. For sequences with match identity percentages that fell below the species-level delimitation thresholds (97% > ITS2 ≥ 85%; 98.3% > 12S ≥ 90%), the matches to GenBank records at the respective percentages were consolidated without making any taxonomic assignments (Data S1).

2.4 | Statistical and ecological analyses

Water samples were grouped categorically to examine differences in coral communities between sample groups via permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) of the *vegan* version 2.5 package (Oksanen et al., 2013) in RSTUDIO version 1.4.1106 (R Core Team, 2021). The sample categories were "depth" (i.e., shallow or deep), "year" (i.e., 2017, 2018 or 2019), "activity" (i.e., spawning or background), "coral cover" (i.e., low or high) and "time" (i.e., day or night). Following verification of homogeneity of dispersion using BETADISPER, we performed PERMANOVA using the adonis function to test whether the above-mentioned categories were associated with coral MOTU compositional differences. Pairwise PERMANOVA tests were conducted using a PAIRWISEADONIS version 0.3 custom script (https://github.com/pmartinezarbizu/pairwiseAdonis), with Bonferroni correction applied for multiple testing. Finally, similarities in coral assemblages across sites were evaluated using the Jaccard similarity coefficient (Jaccard, 1901) and visualized on a nonmetric multidimensional scaling (nMDS) plot.

Read counts from Scleractinia and Actinopterygii were pooled separately within each sample and normalized by the overall sample's sequence read depth. This allowed for estimating and comparing overall relative abundances of the target taxa to track changes between spawning periods and among sampling sites (Laporte et al., 2021).

We assigned trophic levels for each fish species detected with eDNA based on the species metadata on FishBase (Froese & Pauly, 2021). The trophic levels assigned were (i) 2.0–2.5 (lower primary consumers—herbivores), (ii) 2.5–3.0 (upper primary consumers—herbivores and planktivores, (iii) 3.0–3.5 (lower secondary consumers—planktivores and mobile invertebrate feeders [MIFs]), (iv) 3.5–4.0 (upper secondary consumers—omnivores and MIFs), and (v) 4.0–4.5 (tertiary consumers—piscivores and MIFs) (Andradi-Brown et al., 2021; Froese & Pauly, 2021). The fish trophic level pyramid structures of both background and spawning periods in Singapore were compared against those of shallow-water tropical reefs within the Indo-Pacific, specifically from Dampier and Waigeo Islands of Raja Ampat, Indonesia. Briefly, fish species diversity from the Raja Ampat reefs was documented with baited remote underwater video surveys (BRUVS) and, similarly, trophic-level assignments for each species were made with reference to FishBase's diet composition data (Andradi-Brown et al., 2021).

To test for differences in egg sizes of the coral species detected with surveys or eDNA, we first compiled the mean values of egg sizes from the Coral Trait Database (Hartmann et al., 2017; Madin et al., 2016). Subsequently, a literature review of egg sizes for all 71 species was done to complement data from the Coral Trait Database. Specifically, all synonyms of each coral species were compiled from World Register of Marine Species (WoRMS Editorial Board, 2022), and Boolean operators were used to build the search string for Google Scholar search (e.g., "egg size" OR "oocyte size" AND "species name" OR "synonym1" OR "synonym2" etc.). Mean and maximum egg size measurements were analysed separately with a one-tailed *t* test.

3 | RESULTS

We collected 44 seawater samples from two sites from 2017 to 2019 during the spawning window in April (spawning) and also a week before and after the spawning window (background). Each site had contrasting coral cover (Wong et al., 2018), and we were further interested in eDNA's sensitivity to detect spawning in sites with varying coral cover. We sequenced eDNA metabarcode pools targeting coral-ITS2 and vertebrate-12S markers, generating and assembling 369,425,619 paired-end reads in total.

3.1 | Coral eDNA relative abundance and diversity patterns

For all years and sampling sites, we successfully detected an increase in the proportion of coral reads in the spawning window by two- to three-fold and up to five-fold in 2018 (Figure 2a, yellow-coloured backdrop), although the inflation of sequence reads was not consistent throughout the spawning window (Data S1). In contrast, samples in the background window (Figure 2a, grey-coloured backdrop) mostly had close to 0% relative abundance of coral eDNA reads. Correspondingly, we observed large increases in the number of MOTUs during the spawning window. The high coral cover site (Figure 2, red points) generally had much greater increases in MOTU richness than the low coral cover site (Figure 2, blue points) during the spawning window. These coral signals amassed from cnidarian reads formed 0.1–85.1% of the overall read abundances across samples, while the remainder were dominated by uncultured eukaryotes (10.6–98.9%) from nonspecific amplification with the ITS2 marker (Figure S1a; Data S1). When excluding nonmetazoan reads, Scleractinia (1.63–99.2%) and Actiniaria (0.02–96.3%) constituted most of the cnidarian reads (Figure S1b).

Coral eDNA communities were found to be significantly distinct between samples from background and spawning windows (PERMANOVA: $df = 1, F = 1.852, R^2 = .093, p = .042$), as well between the sampling sites (high vs. low coral cover; PERMANOVA: $df = 1$, $F = 1.811$, $R^2 = .054$, $p = .032$) and among years (PERMANOVA: $df = 1$, $F = 3.792$, $R^2 = .102$, $p = .0002$) (Figure 3). There were also significant differences when comparing the night and daytime water samples from 2019 (PERMANOVA: $df = 1$, $F = 4.290$, $R^2 = .263$, $p = .001$); daytime sampling detected up to two times more MOTUs and eDNA signals than night sampling (Day MOTUs ≤14, eDNA spike ≤2.6%; Night MOTUs ≤7, eDNA spike ≤1.4%; Figure 2; Data S1). No

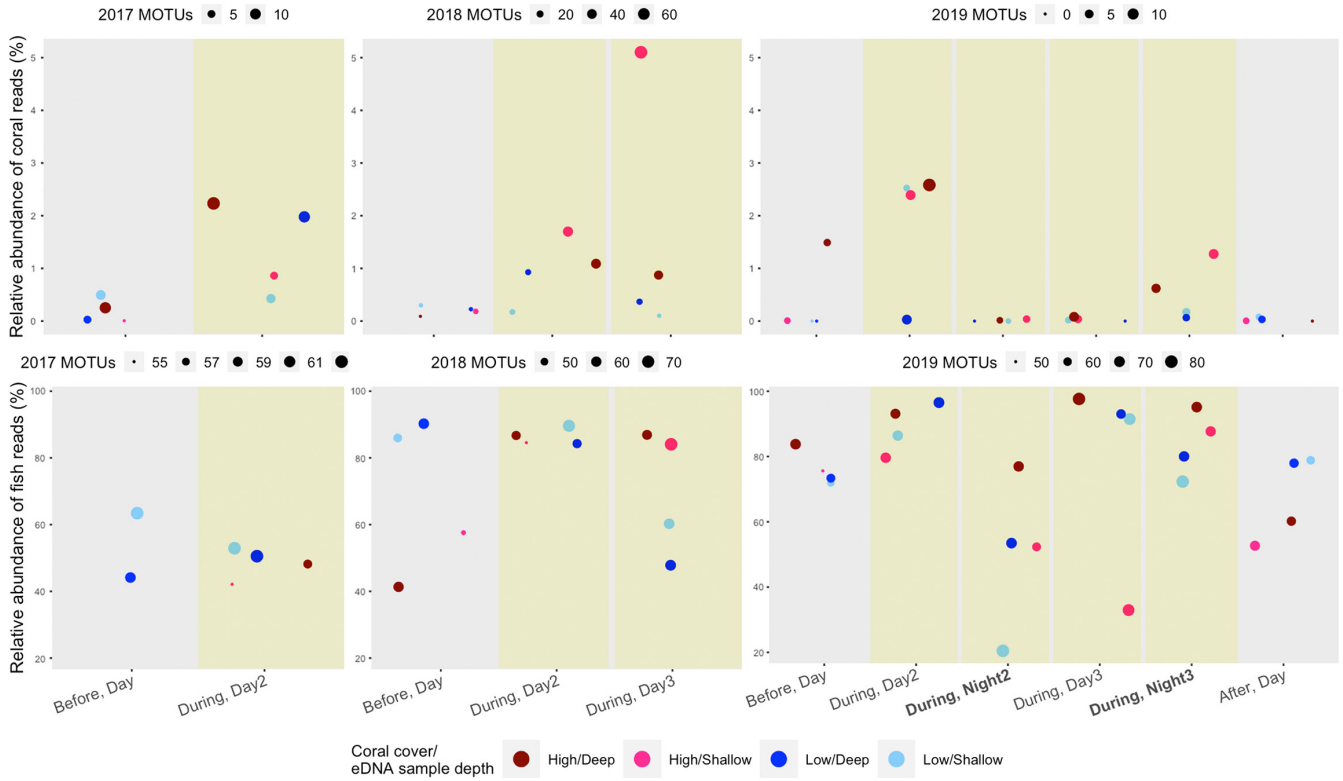

FIGURE 2 Relative abundance of coral ITS2 (upper panel) and fish 12S (lower panel) sequence reads over 3 years. All collections were done in the day, except water samples from the night collections, which are indicated in bold. eDNA collections conducted in the spawning window are indicated by "during" and yellow-coloured background, while those conducted in the background window are labelled as "before" or "after" with grey-coloured background. Point colours represent eDNA signals from shallow, 1 m (lighter shade), or deep, 10 m (darker shade), water samples, generated from low (blue; CYR) and high (red; LSIS: 2017; RLH: 2018 and 2019) coral cover sites respectively. The number of coral MOTUs detected in each sample is indicated by dot size, and the positions have been jittered to reduce overlap for easy visualization.

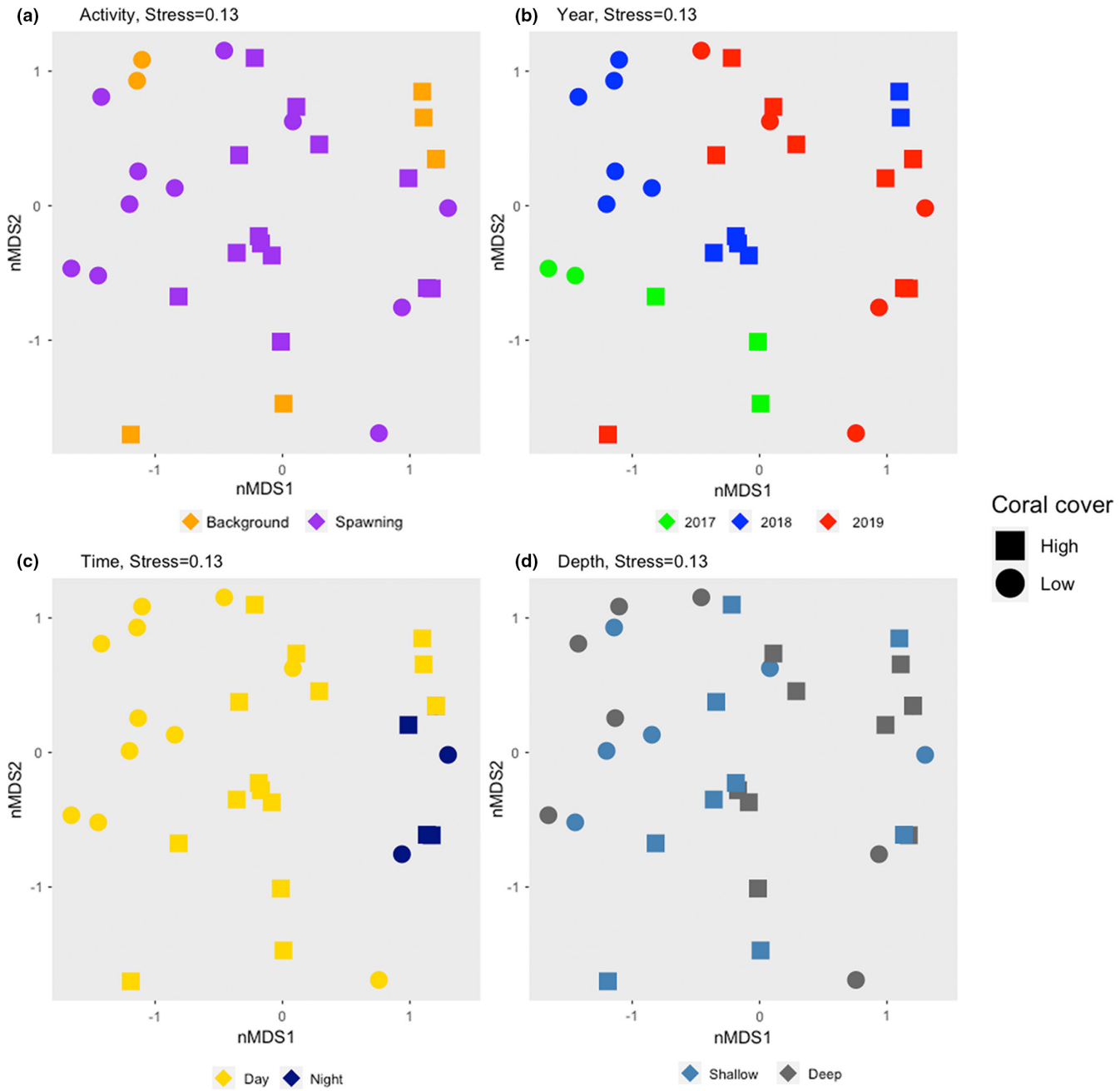

FIGURE 3 Nonmetric multidimensional scaling (nMDS) based on Jaccard similarity among coral MOTU communities detected in each sample using the ITS2 gene marker across high and low coral cover sites, based on the type of activity (a), year (b), sampling time (c) and water collection depth (d).

community differences were apparent between samples collected at 1 or 10 m depth (PERMANOVA: $df = 1$, $F = 1.028$, $R^2 = .054$, $p = .406$).

3.2 | Comparing coral species between eDNA and visual surveys

Taxonomic assignment of coral sequences was performed by applying a species-level delimitation threshold, clustering unique sequence reads into MOTUs (Škaloud et al., 2018). A total of 38 coral MOTUs with species-level identities matched at ≥97% (Afiq-Rosli et al., 2019) to GenBank ITS2 sequence records were found to have elevated relative abundances of sequence reads within the spawning window, while also showing no or very few reads in the background window (Data S1). Of the three study years, the highest number of MOTUs was detected in 2018 (Figure 2). The three coral MOTUs with the highest read abundance across all sites and years were mushroom corals (Fungiidae) *Halomitra pileus*, *Lithophyllon scabra* and *Sandalolitha robusta* (Table S1). There were 15 coral MOTUs without obvious elevated proportions in the spawning window and we therefore considered them background MOTUs (Data S1).

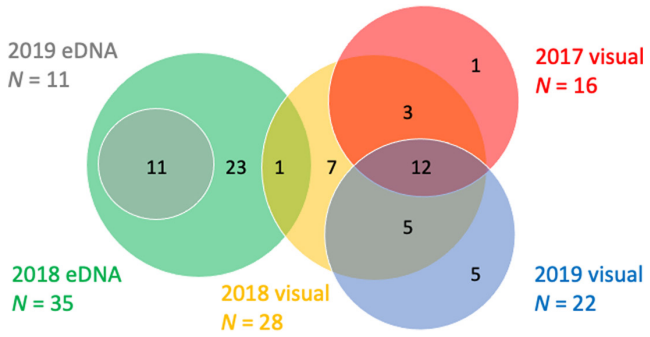

FIGURE 4 Overlapping coral diversity during the spawning window at Raffles Lighthouse (RLH). The visual spawning data were collected over 3 years (red, yellow and blue circles; 2017, 2018 and 2019 respectively) and eDNA metabarcoding experiments with the ITS2 marker over 2 years (green and grey; 2018 and 2019 respectively).

Visual surveys conducted at the high coral cover site (Raffles Lighthouse, RLH) between 2017 and 2019 during the predicted coral spawning window recorded 34 spawning coral species, of which 12 were recorded to spawn every year (Table S1). The highest number of spawning species occurred in 2018 (Figure 4). We found only one overlap between eDNA and visual surveys in 2018—*Goniastrea favulus* (Figure 4; Table S1). Within the 2 years of eDNA sampling at the high coral cover site, RLH, 11 coral MOTUs detected in 2019 overlapped fully with those in the 2018 event (Figure 4). Notably, observed spawning species had >70% overlap between years for the visual surveys (Figure 4). Egg size data for all 71 species detected via eDNA or surveys were searched and compiled from both the Coral Trait Database and 54 other published literature, yielding a total of 195 mean and 39 maximum egg size measurements for 32 coral species, while no information was found for the remaining 39 species (Data S1). The mean egg sizes of spawning species from visual surveys (mean ± *SD* 391 ± 240 μm, $n = 136$) were significantly larger (one-tailed t test, $p < .01$) than those of corals detected by eDNA (302 ± 194 μm, $n = 59$) (Figure S2).

3.3 | Fish eDNA and trophic structure

To test for ray-finned fish eDNA signals that might indicate occurrences of spawning events, we applied a species delimitation threshold of ≤1.7% (Sigsgaard et al., 2020) to cluster Actinopterygii sequences into MOTUs. A total of 133 and 118 fish MOTUs were detected in the spawning and background windows respectively (Data S1), and these constituted the majority (40.9–89.1%) of 12S reads across samples (Figure S1). Temporal trends of both fish eDNA relative abundances and MOTU richness in the background and spawning windows shared limited similarities with coral eDNA signals at the high coral cover site, whereas an inverse relationship was observed for several spawning samples collected from the low coral cover site across all years (Figure 2). Unlike coral MOTU richness trends, which varied considerably between years (Figure 2; 0–60 coral MOTUs), the number of fish MOTUs was more consistent among years, ranging from 50 to 80 (Figure 2). Of the 133 fish MOTUs that showed higher relative read abundances in the spawning window, 19 were consistently detected across all years and sites (Data S1). Several notable secondary (carnivores preying on herbivores) and tertiary (carnivores preying on other carnivores) fish consumers were detected during the spawning window, namely *Abudefduf bengalensis*, *Caranx ignobilis*, *Rachycentron canadum* and *Sphyraena barracuda* (Data S1). Apart from Actinopterygyii, we detected, at lower relative abundances, the presence of several large-bodied vertebrate consumers and predators across the water samples collected during spawning, including sharks and rays (Chondrichthyes; ~11.2%; Ip, Chang, et al., 2021), green sea turtle (~0.69%), dugong (~0.4%), and Indo-Pacific humpback and bottlenose dolphins (~4.1%) (Data S1).

To determine if fish trophic structure differed between the spawning and background windows, we compared the distribution of trophic levels of fish MOTUs/species detected in Singapore during and outside the coral spawning window, to that of two typical shallow-water tropical reefs in Raja Ampat Islands, Indonesia (Andradi-Brown et al., 2021; Figure 5). A bottom-heavy trophic pyramid was evident among the fish communities in Singapore (eDNA survey) and Raja Ampat (shallow-water visual survey) as expected, but an inverted top-heavy pattern was apparent in the spawning window as detected by eDNA in Singapore.

4 | DISCUSSION

To date, eDNA studies on coral reefs have mainly focused on general characterization and biodiversity monitoring (Alexander et al., 2020; Dugal et al., 2021; Nichols & Marko, 2019). In this study, we applied eDNA metabarcoding to dissect coral spawning events and demonstrate that it is feasible to detect signatures of coral spawning from water samples. This finding has broad application across sites of varying coral abundances. Furthermore, we were able to infer the possibility of day–night partitioning of spawning, observe fish trophic structure shifts as indicators of spawning events, and relate coral egg size patterns during the spawning window that demonstrate eDNA's capability in alleviating visual observation biases. In addition to records of spawning participation by multiple coral species, our approach yielded more ecological information within a limited time window, such as inferring predator activity from ray-finned fishes and detecting spawning in species overlooked by surveys, which could be used to study a variety of spatial and temporal patterns during the coral spawning season.

Throughout our 3-year study, we consistently detected elevated levels of coral eDNA and MOTU richness within the spawning window (Figure 2), indicating multicoral species participation in the mass spawning event (Table S1). Trends in eDNA spikes during the spawning window were apparent at both high and low coral cover sites, though increases in coral eDNA were invariably more prominent for the high coral cover site (Figure 2). These results demonstrate the

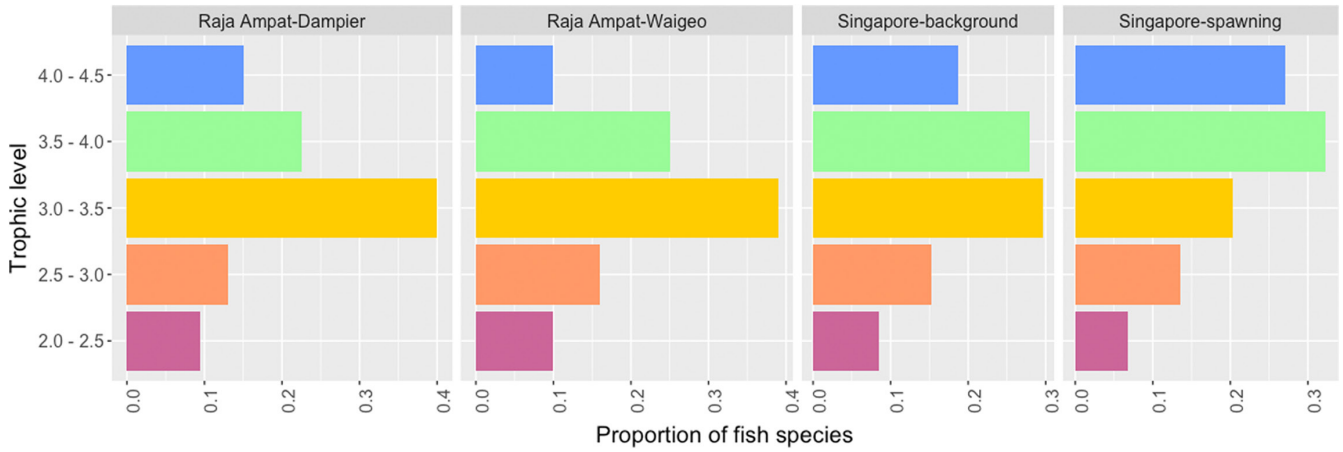

FIGURE 5 Distribution of trophic levels from fish MOTUs/species detected across all the years during the coral spawning event (eDNA; right panel), as compared to the background period in both Singapore (eDNA; middle panel) and two typical shallow-water tropical reefs in Raja Ampat Islands, Indonesia (Andradi-Brown et al., 2021) (baited remote underwater video surveys; left panels). The trophic level groups were (i) 2.0–2.5 (red bars, lower primary consumers—herbivores), (ii) 2.5–3.0 (orange bars, upper primary consumers—herbivores and planktivores), (iii) 3.0–3.5 (yellow bars, lower secondary consumers—planktivores and mobile invertebrate feeders [MIFs]), (iv) 3.5–4.0 (green bars, upper secondary consumers—omnivores and MIFs), and (v) 4.0–4.5 (blue bars, tertiary consumers—piscivores and MIFs). Inverted pyramid or the top-heavy pattern is apparent in Singapore's fish communities detected by eDNA during the coral spawning event but is less and not apparent with the background period in Singapore and Raja Ampat (bottom-heavy) respectively. Trophic levels of the fish MOTUs were obtained from FishBase (https://www.fishbase.in/home.htm).

broad applicability and sensitivity of eDNA as a quantitative tool for monitoring spawning activity at reefs with varying coral abundances. Indeed, we detected 38 named coral MOTUs with elevated proportions of eDNA in the spawning window (Table S1) that were not detected or detected in low relative abundances in the background (Data S1). This suggests that these species were participants in synchronous spawning. Notably, the sampled eDNA coral communities were significantly different between spawning periods, sites and years (Figure 3). While the differences associated with spawning periods and sites were expected, the variation in the number of spawning species between years observed in both eDNA and survey data could be due to asynchronous gametogenesis cycles between species that range between 6 and 14 months (Baird et al., 2009; Mangubhai & Harrison, 2008; Wijayanti et al., 2019). Hence, not every species will spawn consistently every year or within the same spawning window. Relatedly, biannual spawning events have been reported occasionally in Singapore, with some corals spawning in the second and lesser-known spawning window in September (Guest et al., 2005b), resulting in variations in annual species records for the April spawning period. Biannual spawning events and extended spawning periods have also been reported in nearby equatorial (Gouezo et al., 2020; Kenyon, 1995; Penland et al., 2004; Wijayanti et al., 2019) and high-latitude reefs (Baird, Edwards, et al., 2021; Hayashibara et al., 1993) respectively, thus highlighting that more work is needed to ascertain the exact spawning weeks for long-term monitoring and understanding species-specific spawning patterns throughout the year (Foster et al., 2018).

Interestingly, there were no significant differences between the shallow and deep-water samples, even though depth is known to play a major role in the periodicity of coral spawning events (Holstein et al., 2016; Liberman et al., 2018; Prasetia et al., 2017; Shlesinger & Loya, 2019b). We suggest that this could have resulted from temporal sampling limitations, in which eDNA was sampled late after gamete release and most of the coral spawn may have eventually floated to the sea surface. Nevertheless, we observed anomalies such as the elevated relative abundance of coral reads contributed by *Lobactis scutaria* and *Pavona cactus* at 10 m depth in 2017, and before the predicted spawning window in 2019. *Lobactis scutaria* is known to have an extended reproductive season (Kramarsky-Winter & Loya, 1998), and fungiid corals are also reported to produce negatively buoyant eggs that do not form surface slicks (Loya et al., 2009). More generally, we surmise that sampling at the shallow water depth will suffice for broad species detection of nonfungiid corals, whereas targeted species sampling can be done at depth or the midwater column during predicted spawning times.

Visual surveys found 34 spawning species with only one species overlapping with the eDNA survey (Table S1), indicating vastly different assemblages detected between methods (Figure S2). Further verification with visual surveys of species complemented by eDNA sampling is needed for further validation of eDNA's capabilities in species detection and tracking read abundances. The limited overlap between the coral community as assessed by diving and eDNA may have arisen from a combination of field sampling and molecular bench work limitations, stemming partly from the disparity between water sampling and visual surveying times (Data S1), and partially due to amplification biases or nonspecific amplification (Ip, Tay, et al., 2021). The majority of the eDNA samples were collected in the morning after spawning, with the initial goal of optimizing a protocol under daylight conditions to address visibility and safety limitations at night. However, this could have resulted in missed detections of

the spawning signals from the night before due to flushing of gametes by prevailing currents, especially of the species visually observed to spawn before midnight (Data S1). Eventually when eDNA was sampled it would have detected sunrise or midday spawning species. Future studies must implement more robust water sampling plans to be conducted concurrently and throughout night visual surveys to address the mismatch in eDNA collection and visual survey timings. Finer temporal resolution of the coral eDNA spawning data can be attained with hourly collection intervals, which would be expected to help improve the species overlap between methods.

Determining a single best gene marker for the metabarcoding of corals is challenging (Shearer & Coffroth, 2008), but we selected ITS2 as it has been shown to be effective in delimiting closely-related coral species from tissue samples (Forsman et al., 2009; Lam & Morton, 2003). This marker was observed to be more universal and less specific when applied on environmental samples here than when working with tissue samples (Afiq-Rosli et al., 2019), generating wide-ranging proportions (0.1–85.1%) of cnidarian reads across samples (Figure S1). This could have resulted in missed detection of several visually verified spawning species since the remaining reads were dominated by nonspecific amplification of other eukaryotic taxa (Data S1). We thus recommend using additional scleractinian-specific primers to enhance target specificity (Nichols & Marko, 2019; Shinzato et al., 2021). The ITS2 marker may also have insufficient resolution for delimiting the same coral groups that visual methods selectively documented, such as Acroporidae, Lobophylliidae and Merulinidae (Lin & Nozawa, 2017), which we managed to detect with eDNA but could only identify at the family level (Data S1). Species-level identities were unobtainable from sequences for these taxa, as there were multiple GenBank matches at similar high percentages (97–100%) between congenerics (Data S1). Some of the typical taxa targeted in visual surveys (Lin & Nozawa, 2017; Shlesinger & Loya, 2019a) were also not found in our eDNA data set (Table S1), further substantiating insufficient primer resolution that could have contributed to poor matches between eDNA results and commonly documented night-spawning species. Moving forward, the use of a multilocus approach may help address amplification biases and delimitation resolution issues, although coral metabarcodes recovered from improved taxon-specific mitochondrial primers could only be delimited up to genus level (Nichols & Marko, 2019; Shinzato et al., 2021). Other potential workarounds include hybrid-capture approaches to sequence multiple genome-wide loci that have proved successful for species-specific eDNA detection of terrestrial (Seeber et al., 2019) and marine vertebrates (Jensen et al., 2021). Favourably, target-enrichment probes have already been designed for multiple coral species (Quek et al., 2020) and may lead to unambiguous delimitation and more inclusive detection of coral spawning species from environmental water samples.

Species detection with eDNA from shed genetic material should theoretically encompass spawning signals from across all species regardless of coral egg sizes. However, species with larger egg sizes were less frequently captured by eDNA (Figure S2), which may also explain the low species overlap between monitoring methods. We propose two possible remedies to address eDNA's limitations in sampling seawater containing differently sized coral eggs. First, bulk sample DNA metabarcoding techniques can be directly applied to coral gametes. The eggs can be mass collected while dive surveys are ongoing, by either surface horizontal plankton tows (Oliver & Willis, 1987) or from vacuum filtering greater volumes of seawater (~100 L) with the large-pore membranes of Waterra eDNA filters (Peixoto et al., 2021). These mixed sample pools would be subjected to tissue size homogenization, normalization and metabarcoding using a multimarker approach, which could avoid technical biases associated with egg size (Carvalho, 2022; van der Loos & Nijland, 2021). Second, environmental RNA (eRNA) can also be a potential workaround since it comprises shed RNA fragments from upregulation of gene expression in response to biological or environmental stimuli (Yates, Derry, et al., 2021). Spawning signals from eRNA could be more homogenous and recent, potentially reducing false positive detections. Indeed, studies have reported exemplary performances of eRNA in detecting various taxa, including fish (Miyata et al., 2021), polychaetes and tunicates (Wood et al., 2020). However, more coral transcriptome data (Poquita-Du et al., 2019; Quek et al., 2020) and functional genomic work (Cleves et al., 2020) are needed for inferring coral functions from eRNA sequences.

While coral spawning is widely known to occur mostly at night, daytime spawning has been recorded in several species (Eyal-shaham et al., 2019; Kramarsky-Winter & Loya, 1998; Lin & Nozawa, 2017; Mangubhai et al., 2007). Here, spikes in eDNA levels were apparent in both night- and daytime-collected water samples, with possible diurnal partitioning among coral species. We found 30–100% of the MOTUs from daytime samples were from known daytime spawners such as *Pavona cactus* (Mangubhai et al., 2007), *Heliopora coerulea* (Liu et al., 2005), Poritidae (Bronstein & Loya, 2011) and Fungiidae (Eyal-shaham et al., 2019; Mangubhai et al., 2007), which were not detected in the night-time samples. There were slight inconsistencies in the eDNA results, as a mix of signals from both night- and daytime spawners were found in some daytime-collected samples. Specifically, night-time spawners such as *Goniastrea favulus* that were visually observed to spawn at night also had eDNA found in daytime samples. The persistence of night signals in day-collected samples could arise from planulae that remain in the seawater column after fertilization and embryogenesis. Since no data are available for the degradation rates of coral eDNA in Singapore, we were unable to ascertain if the mixed eDNA signatures found in daytime samples were fresh or persistent signals from prior night spawning events. On the one hand, night spawning signals detected during the day could represent persistent eDNA from the night prior to daytime sampling which started as early as 8:30 a.m. (Data S1), especially as temperature and solar radiation exposure are relatively low in the early morning (Strickler et al., 2015). On the other hand, daytime eDNA material faces higher temperatures and more intense solar radiation upon release from host organisms so it manifests as less persistent signals. Future studies should consider temporal effects of water sampling to account for differences in the half-lives of night- and daytime spawning signals. Molecular detection of both

night- and daytime spawning signals here highlights the potential for eDNA to resolve spawning timings relatively efficiently. Apart from collecting water samples in the morning and around midnight, community analyses of water samples can be conducted from two intermediate time frames. For instance, eDNA sampling prior to sunrise (05:00 AM) and in the afternoon (2 PM) can help to distinguish between day and night signals and thus better ascertain the diurnal partitioning of coral spawning.

Apart from higher abundances of fish preying on coral eggs (Pratchett et al., 2001), a temporary shift in trophic structure of fish assemblages with increased presence of predatory fish is also one of the indicators of a spawning event (Heenan et al., 2020; Westneat & Resing, 1988). Our fish eDNA results confirmed this, with spikes in fish eDNA relative abundances and MOTU richness signifying increased fish activity mostly at the high coral cover sites (Figure 2). By contrast, reduced fish eDNA relative abundances on several spawning days were observed at the low coral cover site. Coral spawning events are known to cause spikes in dimethylsulfoniopropionate (DMSP) in the water column (Foretich et al., 2017) due to release of mucus and eggs that contain Symbiodiniaceae endosymbionts (DeBose & Nevitt, 2008). Since DMSP is a known foraging cue for reef fishes (DeBose et al., 2008), we suggest that these highly mobile species could have migrated from the low coral cover site, attracted by the olfactory cues to feed on coral eggs at sites with higher coral cover and more intense spawning activity (DeBose et al., 2008).

Furthermore, there was also an apparent shift in trophic structure during the spawning window relative to baselines on tropical reefs (Figure 5). The observed inverted pyramid pattern indicated increased presence of upper secondary (Figure 5; green bars, trophic levels 3.5–4.0) and tertiary fish consumers (Figure 5; blue bars, trophic levels 4.0–4.5), consistent with expectations of elevated predation levels on coral eggs by planktivorous fish (Pratchett et al., 2001) which are in turn hunted by tertiary predators themselves (Heenan et al., 2020). We found >30% of fish species to be tertiary consumers and, specifically, known secondary consumers of coral eggs such as *Abudefduf bengalensis* (Bengal sergeant). Several notable top reef predators detected during the spawning window were *Sphyraena barracuda* (great barracuda), *Caranx ignobilis* (giant trevally) and *Rachycentron canadum* (cobia) (Data S1). Additionally, there were consistent detections of other large vertebrates, albeit at lower read abundances, which could be attracted to the reef to hunt the large aggregations of fish that feed on coral spawn during the spawning event. These comprised the Indo-Pacific humpback dolphin (*Sousa chinensis*), Indo-Pacific bottlenose dolphin (*Tursiops aduncus*), and requiem sharks such as *Carcharhinus falciformis*, *C. leucas* and *C. melanopterus* (Data S1). These inferences of predatory activity and trophic structure shifts are new applications of eDNA beyond its typical utility in species detection and biomonitoring, which can also be extended to future food web studies with eDNA (D'Alessandro & Mariani, 2021).

To conclude, coral spawning is a complex process that we do not yet fully understand (Baird, Guest, et al., 2021; van Woesik, 2010). However, this study has shown that eDNA metabarcoding can yield more ecological information on other overlooked but spawning-related species within a short span of time to help fill critical knowledge gaps. Specifically, the results underline eDNA's capacity to detect coral spawning signatures with the successful tracking of abundance changes in coral and fish eDNA at both high and low coral cover sites. These new eDNA applications have revealed the possibility of diurnal partitioning of spawning species in Singapore, fish trophic structure shifts and coral egg size patterns, unlocking the potential for applications in food web studies. While molecular tools possess excellent prospects for revolutionizing ecological studies, a better understanding of coral life histories (Madin et al., 2016) remains crucial to enhance study designs and monitoring methods, which will vastly improve detection success via both eDNA and visual surveys.

AUTHOR CONTRIBUTIONS

Y.C.A.I. and D.H. conceived the idea. D.H., K.P.P.T. and R.M. secured funding for the study. Y.C.A.I. and J.J.M.C. collected the water samples. K.P.P.T. led and conducted the visual surveys. K.P.P.T. and Y.C.A.I. compiled the species records, while D.H. verified the species lists. Y.C.A.I. performed the molecular work, data analysis, prepared the figures and drafted the manuscript with input from J.J.M.C., D.H. and R.M.

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CONFLICT OF INTEREST

All authors approved the final version for publication and declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

Raw sequencing reads have been made available at the NCBI Sequence Read Archive under BioProject PRJNA801064.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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