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Mestrado em Biologia Marinha

Supervisors:
Holland Elder
Co-supervisor
Catarina Vinagre

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"I hereby confirm that I have independently composed this Master thesis and that no other than the indicated aid and sources have been used. This work has not been presented to any other examination board."

Abstract

Monitoring threatened ecosystems is critical for understanding and mitigating the ongoing loss of biodiversity. Coral reefs are among the most vulnerable ecosystems, facing numerous threats including climate change, overfishing, and pollution. Restoration activities, such as coral transplantation and the establishment of marine protected areas, are increasingly employed to restore these ecosystems to preserve their ecosystem functions. However, the effectiveness and influence of such restoration efforts on the ecosystem are often difficult to assess due to the challenges of monitoring the vast and complex marine environment.

This study focuses on the use of environmental DNA (eDNA) to monitor coral spawning slicks, which are vital for the natural recovery and maintenance of coral populations. Spawning slicks, composed of gametes and subsequent coral zygotes released during synchronized coral spawning events, provide a unique opportunity to assess the reproductive success and species composition of coral communities. While traditional methods for monitoring restored coral populations are labor-intensive and limited in scope, eDNA analysis of slick samples provides a more efficient alternative. By analyzing eDNA from slick samples collected during spawning events, we can identify the genera present in the slicks, including those that are difficult to detect through visual observations alone.

Utilizing the CoralITS2 and CoralITS2_acro sequencing assays, this study demonstrates the potential of eDNA as a powerful tool for monitoring the composition of coral spawning slicks, thereby providing insights into the reproductive dynamics of restored and at-risk coral species. This approach offers a non-invasive, cost-effective, and scalable method for assessing the success of restoration activities and informing conservation strategies. By integrating eDNA monitoring into existing coral reef management practices, we can enhance our ability to track the recovery of these critical ecosystems and better understand the impacts of restoration efforts.

Keywords

Biodiversity, Coral Reefs, eDNA, ITS2, Metabarcoding, Monitoring

Sumário (Portuguese Abstract)

A monitorização de ecossistemas ameaçados é crucial para entender e mitigar a perda contínua de biodiversidade, especialmente em sistemas vulneráveis como os recifes de corais. Esses recifes enfrentam múltiplas ameaças, incluindo mudanças climáticas, sobrepesca e poluição. Atividades de restauro como o transplante de corais e a criação de áreas marinhas protegidas, estão cada vez mais sendo empregues para restaurar esses ecossistemas à sua saúde original. No entanto, avaliar a eficácia e o impacto desses esforços de restauro é desafiador devido à complexidade e extensão do ambiente marinho. A monitorização das populações de corais e da saúde dos recifes pode ser particularmente difícil, já que a maioria das técnicas tradicionais é invasiva e frequentemente limitada na identificação das espécies presentes.

Este estudo investiga o uso de DNA ambiental (eDNA) como uma ferramenta inovadora e promissora para o monitoramento das manchas de desova de corais, que são eventos críticos para a manutenção e recuperação das populações de corais. As manchas de desova, formadas pelos gametas libertados durante eventos de desova sincronizada dos corais, oferecem uma oportunidade única para avaliar o sucesso reprodutivo e a composição das comunidades de corais. Estes eventos são vitais não apenas para a reprodução dos corais, mas também para a saúde geral dos recifes, que dependem da diversidade genética para se manterem resilientes.

Os métodos tradicionais de monitoramento dessas manchas de desova são trabalhosos e frequentemente limitados no seu âmbito, perdendo muitas vezes espécies que não são visíveis a olho nu ou que são difíceis de detectar com os métodos convencionais. Ao analisar eDNA a partir de amostras de água recolhidas durante eventos de desova, podemos detectar uma gama mais ampla de espécies presentes nas manchas, oferecendo uma visão mais completa da biodiversidade que compõe os recifes de corais.

A metodologia deste estudo envolveu a coleta de amostras das manchas de desova em quatro locais representativos ao longo da Grande Barreira de Coral (GBR) durante as temporadas de desova de 2018 e 2021. Os locais de coleta foram Backnumbers Reef, Keeper Reef, Lizard Island e Whitsundays. As amostras foram recolhidas da superfície das manchas e processadas para extração de DNA. Para validar a eficácia dos ensaios, foram preparadas misturas conhecidas de larvas de dez espécies de corais. Essas misturas permitiram uma avaliação rigorosa das capacidades de detecção dos ensaios e a precisão das razões de abundância relativa dos produtos de metabarcoding.

O ensaio CoralITS2 visou a resolução a nível de género, enquanto o ensaio

CoralITS2_acro foi utilizado porque estudos anteriores mostraram que o primer CoralITS2 não detecta Acropora, um gênero dominante nos recifes da GBR. Essa escolha foi fundamental para melhorar a detecção de Acropora, cuja região ITS2 é mais curta e apresenta dificuldades para a ligação do primer no CoralITS2.

A extração e análise de eDNA foram realizadas com controles rigorosos em laboratórios dedicados para minimizar a contaminação de DNA não amostral. O procedimento envolveu um protocolo de extração de DNA modificado para amostras larvais, incluindo uma incubação de 2,5 a 3 horas em solução tampão e RNase. O DNA extraído foi submetido a dois ensaios de metabarcoding, com amplicons de aproximadamente 419 bp para CoralITS2 e 445 bp para CoralITS2_acro. A escolha do CoralITS2_acro, com o seu primer reverso modificado, foi motivada pela necessidade de melhorar a detecção de Acropora e outros géneros de corais.

Os resultados revelaram um total de 191 Variantes de Sequência de Amplicon (ASVs) identificadas através dos ensaios CoralITS2 e CoralITS2_acro. A criação de misturas conhecidas permitiu verificar as capacidades de detecção de cada ensaio e a precisão das razões de abundância relativa observadas. Embora tenha sido observada uma variação significativa nas razões de abundância relativa entre os replicados, a análise Bayesiana usando um modelo beta-binomial indicou que as abundâncias observadas eram consistentes com os valores esperados, sem desvios significativos detectados. Isso confirma a precisão dos ensaios na reflexão das composições das comunidades de corais e reforça a confiabilidade do eDNA como ferramenta de monitoramento.

O estudo também destacou um trade-off entre a resolução detalhada a nível de gênero e a representação abrangente da diversidade devido às limitações atuais nos bancos de dados genômicos de corais. O ensaio CoralITS2 forneceu uma resolução a nível de gênero para vários gêneros, mas teve limitações na detecção de Acropora. Em contraste, o CoralITS2_acro, com seu primer modificado, conseguiu identificar Acropora juntamente com outros gêneros. A Análise de Coordenadas Principais (PCoA) e outras métricas de distância, como Unweighted UniFrac e Bray-Curtis, revelaram padrões distintos de agrupamento entre as amostras de manchas de desova, refletindo variações na composição das comunidades de corais.

Os dados sugerem que a integração de várias técnicas de metabarcoding é valiosa para alcançar uma compreensão mais completa da diversidade de corais. A abordagem de eDNA provou ser uma ferramenta não invasiva, económica e escalável para monitorar a composição das manchas de desova de corais. Sua capacidade de detectar e identificar espécies que são difíceis de observar visualmente oferece um método mais robusto para avaliar o sucesso da restauração e informar estratégias de conservação. Ao incorporar o monitoramento de eDNA nas práticas existentes de gestão de recifes de corais, podemos aprimorar a nossa capacidade de acompanhar a recuperação desses ecossistemas críticos e obter uma melhor compreensão dos impactos dos esforços de restauro.

Este estudo reafirma o potencial do eDNA como uma ferramenta poderosa para a monitoração de comunidades de corais e destaca a importância das técnicas de metabarcoding integradas para uma avaliação detalhada da biodiversidade. A capacidade de acompanhar a dinâmica reprodutiva dos corais e a diversidade das comunidades oferece novos insights sobre a saúde dos recifes e a eficácia das iniciativas de conservação e restauro. Adotar o eDNA como prática padrão de monitoramento pode melhorar significativamente a nossa compreensão e proteção dos recifes de corais, promovendo a sustentabilidade e recuperação desses ecossistemas vitais. Esta abordagem inovadora representa um avanço significativo na monitorização ambiental e tem o potencial de transformar a forma como avaliamos e gerimos a saúde dos recifes de corais.

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List of abbreviations, acronyms, and symbols

DNA– deoxyribonucleic acid

eDNA – environmental DNA

PCR – Polymerase Chain Reaction

ASV – Amplicon Sequence Variant

OTU – Operational Taxonomic Unit

LCA – the lowest common ancestor

PCoA – principal coordinate analysis

ITS2- Internal Transcribed Spacer 2

Chapter 1 – General Introduction

1.1 Study System

Coral reefs are among the most biologically diverse and valuable ecosystems on the planet, serving as critical resources for human societies, providing essential ecological services, and supporting the livelihoods of millions (UN Environment, 2018). Corals, marine invertebrates that form the foundation of coral reefs by secreting calcium carbonate skeletons, support a vast array of marine life and play a crucial role in maintaining biodiversity (Maragos et al., 1996) and coastal protection (Pascal et al., 2016).

Coral reef ecosystems are highly productive and economically valuable (Fezzi et al., 2023). They support diverse species assemblages, offer flood protection (Beck et al., 2018; Reguero et al., 2021), and drive local economies through tourism (Spalding et al., 2017). Coral reefs are diversity hotspots (Roberts et al., 2002), because coral cover is related to habitat volume and complexity and is a good predictor of fish and invertebrate abundance (Dustan et al., 2013; Idjadi & Edmunds, 2006). Biodiverse coral reef ecosystems provide numerous services through fisheries, and tourism (Arkema et al., 2015), supporting the life of more than half a billion people (UN Environment, 2018), on average providing $350,000/ha per year (Costanza et al., 2014).

Corals are marine invertebrates belonging to the phylum Cnidaria and the class Anthozoa. They comprise small, individual animals called polyps, usually a few millimeters in diameter (Wells, 1956). Scleractinia species, commonly known as stony corals, are primarily known for their role in forming coral reefs by building calcium carbonate skeletons that form the reef, and these are among the most diverse and productive ecosystems on the planet. Scleractinia corals are most abundant in coastal areas between 30° north and -30° south, with the most diversity in coral species concentrated in the western Indian Ocean and on the north coast of Australia (Figure 1.1; Kusumoto et al., 2020).

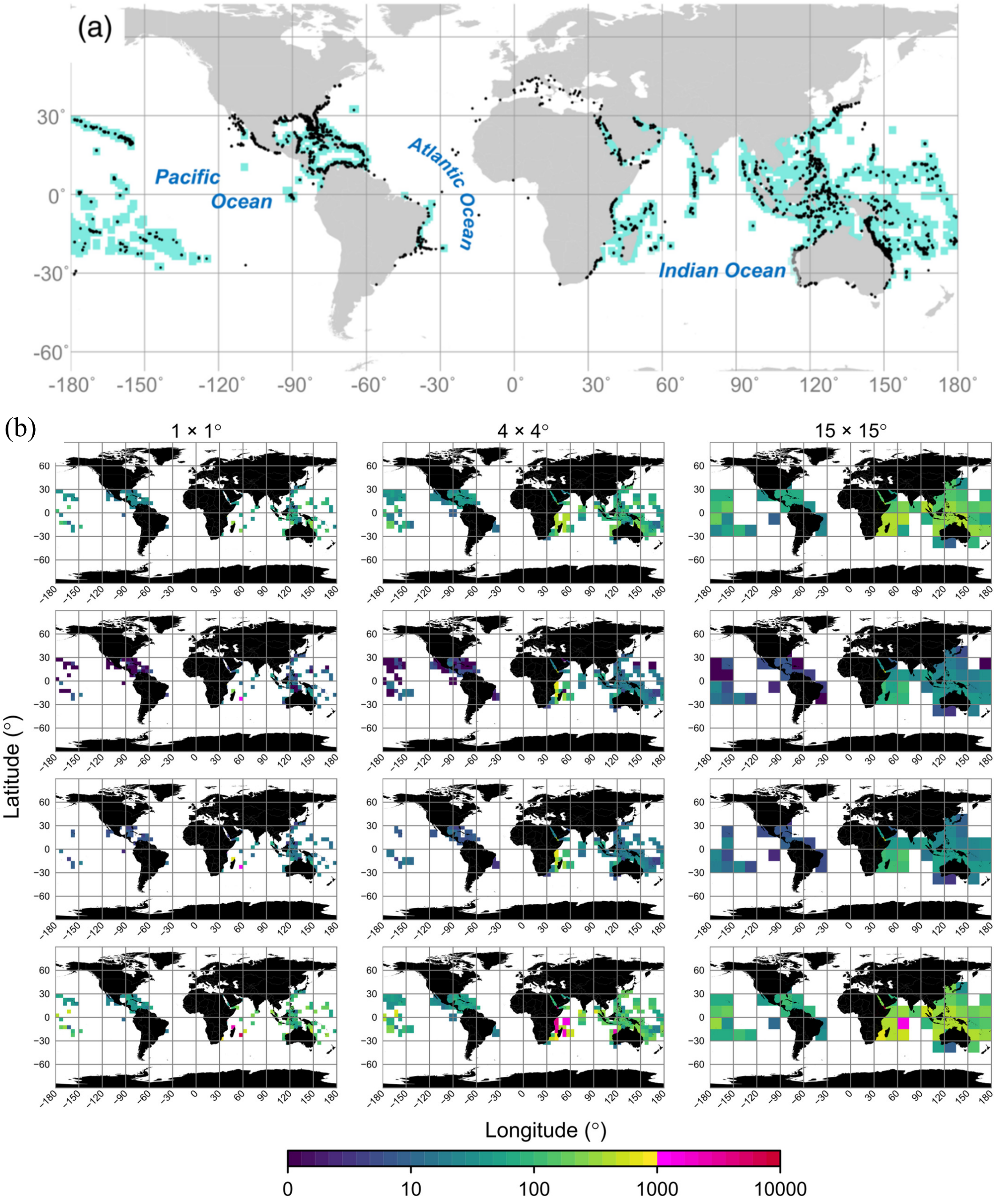


Figure 1.1 Distribution and diversity of Scleractinia corals (a) Occurrence records of coral species of the Scleractinia order, derived from the Global Biodiversity Information Facility and Ocean Biogeographic Information System (109,367 points). Light-blue areas represent the coral reef distribution provided by ReefBase. (b) Geographical patterns of coral species diversity (Hill's number of order $q = 0$) at different spatial resolutions (1, 4 and 15° cells). Diversity is estimated at four sampling coverages (SC): observed SC ($^0D_{ref}$) (top row), 1% percentile of SC values of doubled sample size ($^0D_{SC1}$) (second from the top row), 5% percentile of SC values of doubled sample size ($^0D_{SC5}$) (third row) and infinite SC (i.e., asymptote diversity; $^0D_{asym}$) (bottom row). (**http://www.reefbase.org/main.aspx**). Adapted from Kusumoto et al., 2020.

1.2 Climate Change

The greatest threat to coral reefs is ocean warming caused by anthropogenic greenhouse gas emissions (Hoegh-Guldberg et al., 2019). In particular marine heatwaves, driven by these rising temperatures, threaten global biodiversity as extreme warming events have impacted critical foundation species such as corals, seagrasses, and kelps endangering these critical ecosystems (Smale et al., 2019). The prolonged high temperatures associated with marine heat waves are a key driver of coral bleaching, placing immense stress on coral reefs (Hughes et al., 2017; Intergovernmental Panel on Climate Change (IPCC), 2023). Bleaching can occur as a response to many external stressors, such as high irradiance, darkness, pathogens, and as mentioned heat shock (Douglas, 2003). While bleaching does not only pertain to corals, coral bleaching is the term used to describe the loss of coral colony color, through the partial or total expulsion of the coral-algal symbionts of the family Symbiodiniaceae (Douglas, 2003; Glynn, 1991; LaJeunesse et al., 2018). Without these symbionts, corals are unable to meet their energy needs, which can lead to starvation and eventual death if temperatures do not return to normal for the given reef in time for recovery (Baker et al., 2008; Muscatine, 1990).

While coral bleaching events do not always result in mass coral mortality, the increasing severity and frequency of these events will result in increasing coral mortality and reef degradation outlined in climate change scenarios by the International Panel on Climate Change (Gadoutsis et al., 2019; Intergovernmental Panel on Climate Change (IPCC), 2023; Maynard et al., 2008; Weis, 2010; Yadav et al., 2023). Marine heatwaves have triggered four mass coral bleaching events on the Great Barrier Reef in just seven years, reducing shallow water coral reefs by as much as 50% (Dietzel et al., 2021; Hughes et al., 2017). Given the widespread habitat degradation, there is a growing acknowledgment that active restoration efforts are essential to ensure the continued viability of natural ecosystems and the services they provide in the medium-term future (Bullock et al., 2011).

1.3 Coral Reproduction

1.3.1 Asexual versus sexual reproduction

Scleractinia corals have a biphasic life cycle consisting of a dominant sessile, benthic polyp phase, and a motile planula larval phase (Harrison, 2011; Harrison & Wallace, 1990; Richmond & Hunter, 1990). Polyps, the basic units of a coral colony, primarily engage in somatic growth and asexual budding, which produces new, genetically identical polyps. Once a colony attains sufficient size and age, it may enter a reproductive cycle (Harrison, 2011; Harrison & Wallace, 1990; Richmond & Hunter, 1990). Corals reproduce sexually through two primary modes: brooding, where fertilization occurs internally and larvae are released over prolonged periods, and broadcast spawning, where eggs and sperm are released simultaneously into the water column, with external fertilization typically occurring during a mass synchronized spawning event (Fadlallah, 1983; Harrison, 1985; Madin et al., 2016; Richmond & Hunter, 1990). Synchronously spawning corals may release billions of sperm and positively buoyant eggs per hectare of reef (Álvarez-Noriega et al., 2016; Howells et al., 2016; Willis et al., 1985), which can create highly concentrated and species-rich surface slicks (Butler, 1980; Oliver & Willis, 1987). The species composition and viability of the slick are strongly influenced by the abundance and composition of adult coral species on the reef, particularly from the most fecund families. Approximately half of the 900 extant species of hermatypic scleractinians have identified modes of sexual reproduction (Veron JV, 2000), and the diversity of documented reproductive patterns will likely continue to expand as more taxa are studied (Randall et al., 2020).

1.3.2 Reproduction cycle factors

Coral reproductive cycles are intricately tied to environmental conditions that impact their survival and fitness (Randall et al., 2020). These conditions can be categorized into ultimate and proximate factors. Ultimate factors exert long-term evolutionary pressures to maximize reproductive success (Harrison & Wallace, 1990). They include seasonal temperature changes, which optimize physiological performance (Babcock & Heyward, 1986; Keith et al., 2016), wind speeds that affect fertilization and dispersal (Heyward & Negri, 2012), tidal phases that influence fertilization success (Babcock & Heyward, 1986), and diurnal cycles that help in avoiding predators (Harrison et al., 1984).

On the other hand, proximate factors are short-term environmental cues that synchronize and regulate reproductive events more immediately. These factors include annual, seasonal, lunar, and daily cues, which interact with the corals' endogenous biorhythms to optimize reproduction (Harrison & Wallace, 1990; Hoadley et al., 2016). These involve environmental daily cues such as lunar effects (Hoadley et al., 2016). Each of these factors plays a crucial role in shaping the reproductive success of coral populations over time.

1.3.3 Coral Spawning

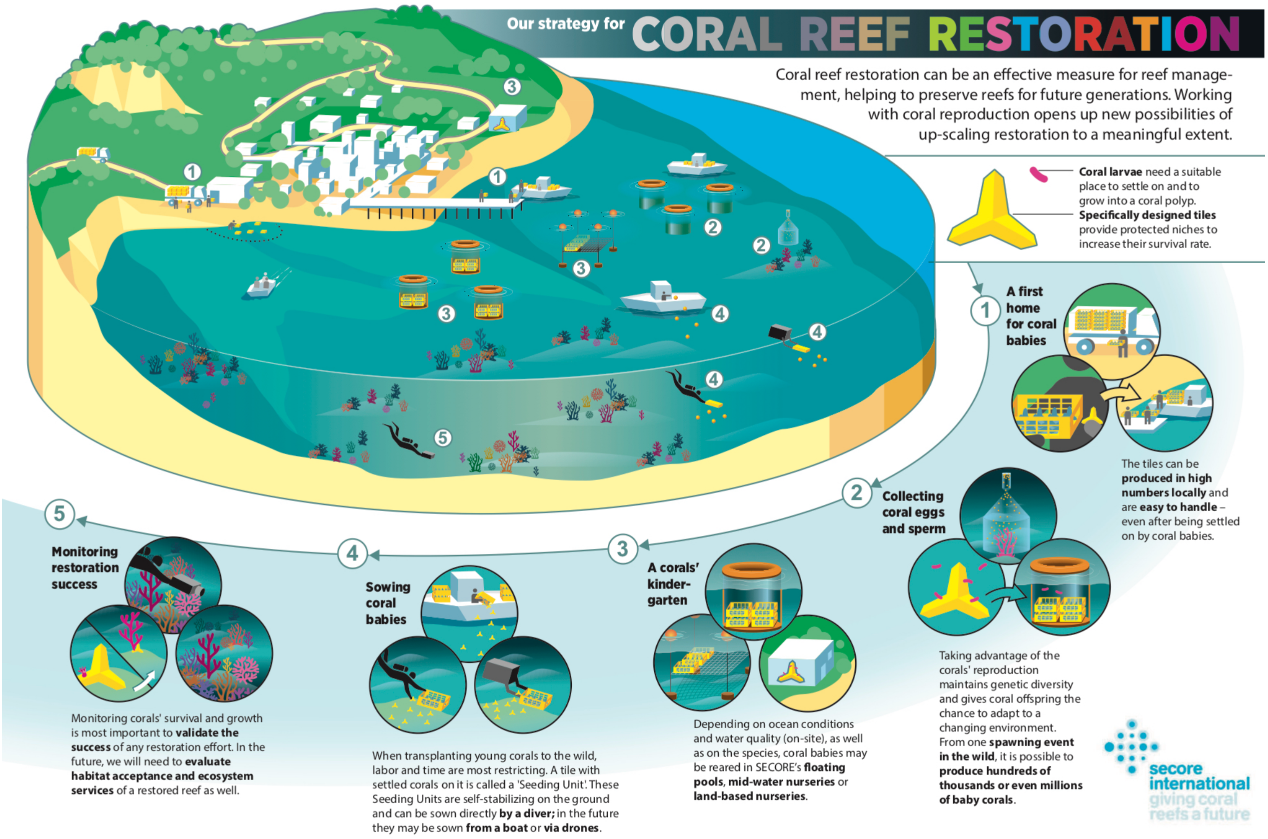
Spawning in corals typically occurs once a year during a synchronized event, where many individuals release gametes simultaneously. However, this process can also be protracted, occurring as sequential spawning events over several months or even across different seasons. Studies have shown that spawning can be spread over an extended period, highlighting the variability in reproductive timing among coral populations. In certain locations, biannual spawning has been documented, indicating that some coral populations reproduce more frequently (Bouwmeester et al., 2016; Stobart et al., 1992). Additionally, a phenomenon known as split-spawning, which occurs roughly every 2 to 3 years, involves either a single colony spawning over 2 to 3 consecutive months or different colonies from the same population spawning in successive lunar cycles (Baird et al., 2009). Identifying corals contributing to various spawning patterns and understanding these diverse reproductive patterns is essential for developing effective coral restoration strategies and ensuring the health and resilience of coral reef ecosystems.

1.4 Coral Restoration Techniques

1.4.1 Review of techniques

Coral restoration efforts seek various goals but commonly aim to revive damaged ecosystems, enhance their resilience to ongoing environmental stresses, and ensure these environments continue to provide essential ecological, economic, and cultural benefits. Methods for restoration of coral reefs are composed of two main techniques: asexual and sexual reproduction. Both techniques have two main phases (1) propagation and nursery farming and (2) transplantation/out plantation. Many coral restoration initiatives have utilized asexual propagation 'coral gardening' techniques, which is where corals are fragmented or micro-fragmented and out-planted onto reefs (Forsman et al., 2006, 2015; Lirman & Schopmeyer, 2016; Rinkevich, 2005, 2008; Young et al., 2012). However, this technique yields low genetic diversity which constrains outplants' opportunity for fertilization and resistance to disease and future stress disturbances (Barton et al., 2017; Beeden et al., 2008).

In contrast, restoring degraded reefs with sexually propagated corals has a strong potential to increase reef genetic diversity, and increase coral heat tolerance (Evensen et al., 2021; Howells et al., 2021; Morikawa & Palumbi, 2019; Voolstra et al., 2020), and be upscaled to more cost-effective processes. One approach involves mass coral larval enhancement ('reseeding'), which rears and settles millions of larvae from large-scale spawning events of highly fecund corals (Babcock & Heyward, 1986; P. L. Harrison & Wallace, 1990; R. L. Harrison et al., 1984). This method not only provides a more cost-effective solution but also offers the advantage of greater genotypic diversity among recruits, likely improving adaptive potential, resistance to future disturbances, and overall coral community resilience and recovery rates (dela Cruz & Harrison, 2020; Van Oppen et al., 2015). The basic approach for sexual propagation is to (1) create settlement tiles that are easy to handle (2) collect coral eggs and sperm from spawning events (3) raise settled coral to a certain size/ age in floating pools, mid-water nurseries, or land-based nurseries (4) transplant seeding devices into the wild (5) monitor restoration success (Figure 1.2).



Coral reef restoration can be an effective measure for reef management, helping to preserve reefs for future generations. Working with coral reproduction opens up new possibilities of up-scaling restoration to a meaningful extent.

Coral larvae need a suitable place to settle on and to grow into a coral polyp. **Specifically designed tiles** provide protected niches to increase their survival rate.

The tiles can be **produced in high numbers locally** and are **easy to handle** – even after being settled on by coral babies.

Taking advantage of the corals' reproduction maintains genetic diversity and gives coral offspring the chance to adapt to a changing environment. From one **spawning event in the wild**, it is possible to **produce hundreds of thousands or even millions of baby corals**.

Depending on ocean conditions and water quality (on-site), as well as on the species, coral babies may be reared in SECORE's **floating pools**, **mid-water nurseries** or **land-based nurseries**.

When transplanting young corals to the wild, labor and time are most restricting. A tile with settled corals on it is called a 'Seeding Unit'. These Seeding Units are self-stabilizing on the ground and can be sown directly **by a diver;** in the future they may be sown **from a boat** or **via drones**.

Monitoring corals' survival and growth is most important to **validate the success** of any restoration effort. In the future, we will need to **evaluate habitat acceptance and ecosystem services** of a restored reef as well.

Figure 1.2 Illustration of SECORE's up-scaling coral reef restoration strategy utilizing sexually reproduced coral. (see secore.org)

1.4.2 Monitoring and Evaluation

Monitoring restoration activities is essential to assess the method's effectiveness in meeting the restoration goals. Restoration monitoring of corals to date has revolved around evaluating survival and growth of transplants through photographs and diver surveys, and maintenance such as cleaning (removal of coral predators), and repairs (reattaching fragments), but most monitoring efforts were not adequate to evaluate long term success (Ferse et al., 2021). Consistent, comparable, and quantitative data is required to quantify the changes that result from restoration activities and ensure that the goals of the restoration work are being achieved.

If the goal is to restore the ecosystem, we need additional metrics to evaluate whether transplanted corals are sexually reproducing and tracking the repopulation of reefs. It is essential to investigate when transplants reach sexual maturity and quantify the contribution of species' genetic material to the relative abundance in spawning slicks, as these pools of genetic material become the source of larvae populating the reef in the subsequent generations. Additionally, selecting coral slicks with high genetic diversity and significant larval contributions will enhance the reseeding restoration efforts previously discussed. While there are current practices for investigating which corals are contributing to mass spawning slicks, these efforts are labor intensive, involving surveys and the identification of adult morphotypes based on their morphological traits. Achieving accurate results with this kind of approach requires considerable taxonomic expertise (Goldstein & DeSalle, 2011). Even with skilled taxonomists, some species may be overlooked because it is rarely feasible to conduct intensive surveys of spawning adult corals over large reef areas, resulting in studies that may not fully capture the diversity of spawning corals. Lastly, as SCUBA diving is a common method for conducting such surveys, they are limited by time, depth, and inherent risks, and are additionally invasive during spawning events. As a result, environmental DNA (eDNA) metabarcoding is being tested as an alternative method to traditional surveys to improve the detection and accuracy of monitoring restoration activities.

1.5 Environmental DNA (eDNA)

1.5.1 eDNA overview

Environmental DNA (eDNA) refers to the DNA extracted from various environmental samples, including soil, sediments, water, or snow. In aquatic and semi-aquatic environments, eDNA has been used to monitor populations by analyzing DNA collected from water bodies. This DNA can come from a variety of sources, such as feces, saliva, urine, and skin cells of animals living in the water, as well as from animals like birds and mammals that visit the water. Analyzing water for species-specific eDNA is a new and developing technique with promising applications for surveying aquatic organisms and supporting conservation efforts.

The use of environmental DNA (eDNA) analysis for monitoring and conserving aquatic populations originated from methods employed in diversity assessments in ancient sediments (Willerslev et al., 2003). The first eDNA study on freshwater samples focused on detecting the invasive American bullfrog (Ficetola et al., 2008). Since then, eDNA has been used to monitor a wide range of species, including amphibians (Goldberg et al., 2011), fishes (Jerde et al., 2011; Thomsen et al., 2012), mammals (Foote et al., 2012), and corals (Alexander et al., 2020; Dugal et al., 2022; West et al., 2022). These studies typically involve detecting short fragments of mitochondrial DNA (mtDNA), which are preferred due to their higher copy number compared to nuclear DNA, especially in environments where DNA concentrations are low or degraded. Efforts have been made to develop short-sequence DNA barcodes for species identification, which are crucial for environmental monitoring (Hebert & Gregory, 2005). eDNA workflows can typically be divided into main steps (1)- fieldwork, where sample collection, filtering, and preservation take place (2)- laboratory processing for DNA extraction, amplification, and sequencing and (3)- data processing where results from lab work are analyzed, taxonomy assigned, and statistical analysis takes place (Figure 1.3).

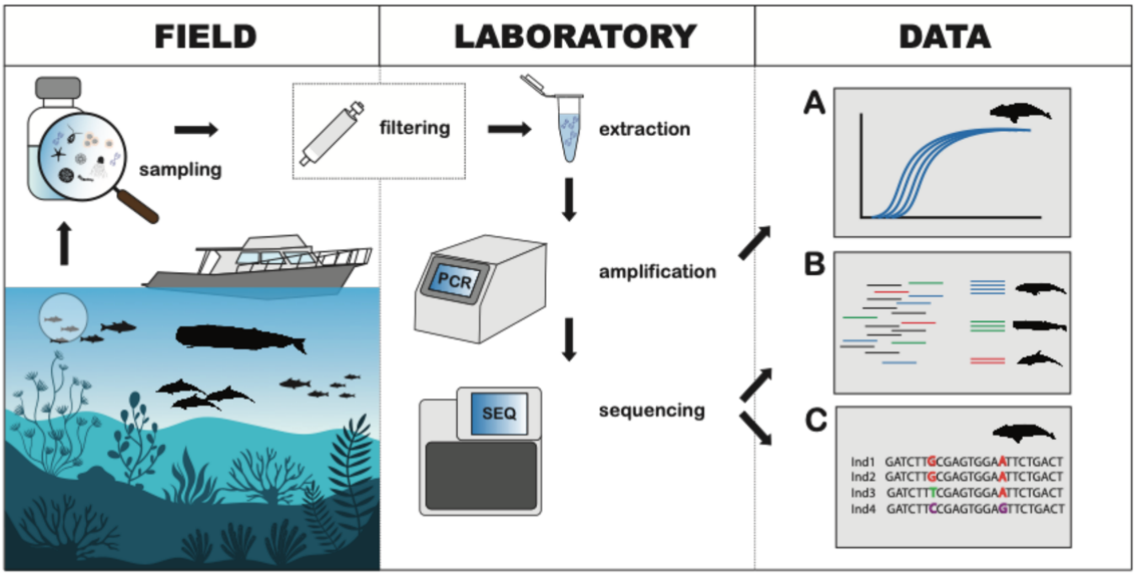


Figure 1.3 Schematic overview of the workflow associated with eDNA sampling, sample processing and data generation options. Samples either in the field or in a laboratory. The "DATA" panel explains the commonly used methods to study marine mammals with eDNA, including A) absolute quantification of the presence of DNA from target species with e.g. qPCR, B) metabarcoding for species detection and community inference, and C) the identification of SNPs and haplotype sequencing for population genetics. Adapted from Székely et al., 2022.

Sampling for eDNA has varied from 15mL (Ficetola et al., 2008) to 10L of water from the given environment (Goldberg et al., 2011), and sample sizes increase depending on the likelihood of detecting low-abundant species (Goldberg et al., 2011). The probability of eDNA detection varies based on the target species and is influenced by the density of the organisms in the environment, the specific taxa being studied, and the water body being sampled (Rees et al., 2014). Due to target detection and variation in PCR bias, the number of biological replicates commonly used ranges from 4-12 (De Barba et al., 2014; Ficetola et al., 2015). Samples are processed using various DNA extraction methods before proceeding to metabarcoding. Metabarcoding builds on the concepts of traditional molecular barcoding

(Hebert et al., 2003) and extends them to multi-species samples using Next Generation Sequencing. In this process, genetic material from the samples is extracted, and the DNA is then amplified through metabarcoding assays. This technique enables the simultaneous, high-throughput sequencing of specific barcode regions, which can be identified to a taxonomic level using a reference database like GenBank or analyzed independently of taxonomy as operational taxonomic units (OTUs; Shokralla et al., 2012).

Relative abundance estimations from metabarcoding results are possible but there are limitations to consider (Figure 1.4). Although PCR is a powerful tool in metabarcoding studies, it can introduce biases that may distort diversity estimates. These biases can affect taxa abundance estimations and include factors such as annealing temperature, template secondary structures (Fonseca et al., 2012), primer-template mismatches, taxon-specific amplification bias (variation in target sequence length), variation in the copy number of target loci, and amplicon size (Figure 1.4; Császár et al., 2010; Fonseca, 2018). Additionally, if the metabarcoding assay of choice fails to detect certain taxa, the relative abundance of detected groups will be overestimated.

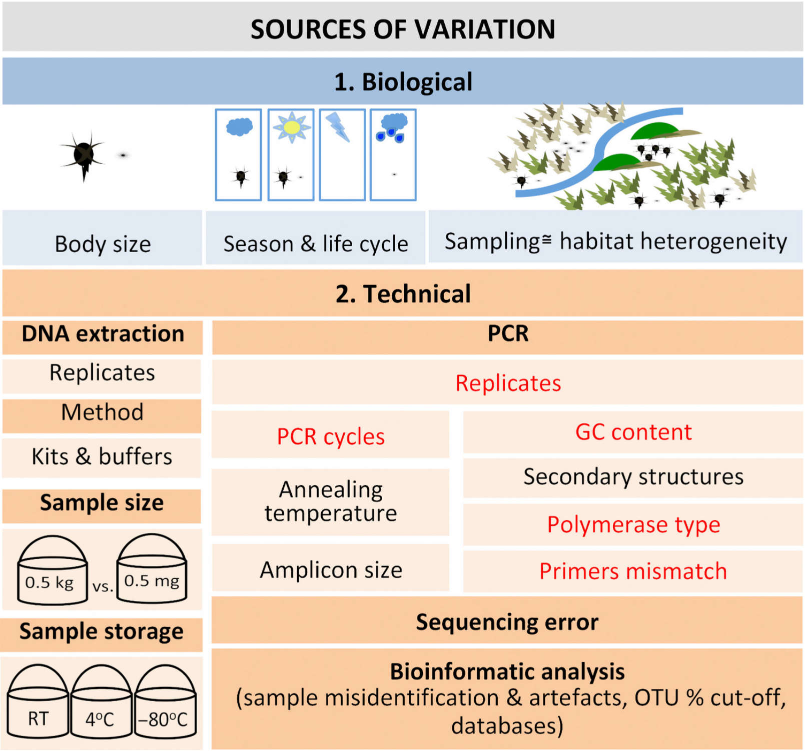


Figure 1.4 Variation in eDNA diversity estimates considering biological to technical sources. Adapted from (Fonseca, 2018)

Imperfect detection is an inherent challenge in most data collection efforts related to species presence, absence, and abundance. Even in traditional ecological field studies, not all individuals or species present at a site are always detected, and failing to account for this imperfect detection can lead to biased conclusions (Kéry & Schmidt, 2008; Yoccoz et al., 2001). A crucial step in validating the presence and abundance data in metabarcoding studies involves testing PCR primers on known samples to verify successful PCR amplification and accurate taxonomic assignment from subsequent steps. The CoralITS2 and CoralITS2_acro primers identified for this study have been previously evaluated for species detection using coral samples from Western Australia reef sites, as recorded in library records. However, these primers were tested on only a single sample per species (Dugal et al., 2022). Previous detection results of the CoralITS2 and CoralITS2_acro assays on wild samples (Alexander et al., 2020; Dugal et al., 2022; West et al., 2022) could be leveraged to inform prior estimations of expected and observed relative abundance ratios of a known mix.

1.5.2 Primer selection

To detect organisms using environmental DNA (eDNA) methods, specific primers are required to bind to the target organisms' DNA, enabling successful PCR amplification. The CoralITS2 assay primers have been used in previous studies for coral detection (Brain et al., 2019). However, it has been demonstrated that the CoralITS2 reverse primer was ineffective in binding to members of the genus *Acropora* (Alexander et al., 2020), which dominates many sites on the Great Barrier Reef (Done, 1982). To address this issue, an alternative reverse primer was developed specifically to target *Acropora* while complementing the existing CoralITS2 forward primer. This newly designed reverse primer binds to the 28S gene region, which has high homology across *Acropora* species (Alexander et al., 2020).

The 28S region is part of the 28S ribosomal RNA (rRNA) gene, a component of the large subunit of eukaryotic ribosomes. This gene is highly conserved across many species, making it a common target in molecular biology for phylogenetic studies and species identification, including in eDNA research (Machida & Knowlton, 2012). Despite its overall conservation, certain regions of the 28S gene exhibit variability that can distinguish between taxonomic groups, making it a valuable marker for identifying specific taxa (Wei et al., 2006). Its slow rate of variation over evolutionary timescales also makes it useful for studying evolutionary relationships (Hillis & Dixon, 1991).

1.6 Statistical Analysis of Ecological Data

Statistical analysis of ecological data is a crucial component in understanding complex environmental interactions and informing conservation efforts. Traditional frequentist statistics, which rely on hypothesis testing and confidence intervals, have long been the standard approach for analyzing ecological datasets, providing a robust framework for assessing patterns and relationships. However, with the increasing complexity of ecological data, alternative methods such as Bayesian models and multivariate techniques have gained prominence. Bayesian models offer a flexible approach by incorporating prior information and updating it with new data, allowing for more nuanced inferences in ecological studies. Additionally, multivariate analyses like Principal Coordinate Analysis (PCoA) and various distance metrics- such as Unweighted UniFrac, Weighted UniFrac, Bray-Curtis, and Jaccard- enable researchers to explore patterns of community composition and diversity across different ecological gradients. These advanced statistical tools allow for a deeper understanding of biodiversity, species distributions, and ecological processes, ultimately contributing to more effective conservation and management strategies.

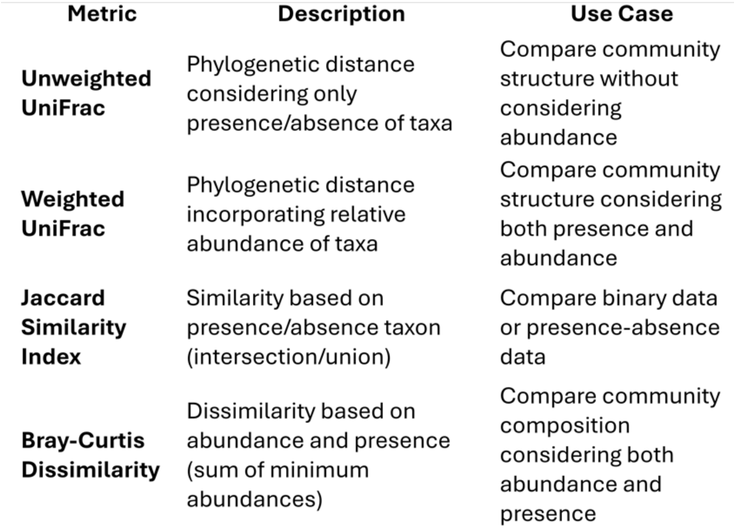
1.6.1 Frequentist versus Bayesian

Frequentist and Bayesian approaches to statistical analysis differ significantly in their methods of testing, decision-making, estimation, and uncertainty quantification. Frequentist statistics, which include methods like p-values for hypothesis testing, introduced by Fisher in 1925 (Fisher, 1992) and error probabilities developed by Neyman and Pearson in 1928 (Neyman & Pearson, 1928), rely heavily on null hypothesis significance testing (NHST). In contrast, Bayesian statistics use Bayes Factor, a measure introduced by Jeffreys in 1961, to compare the likelihood of one hypothesis over another (H_0 versus H_1)(Jeffreys, 1961). When it comes to estimation and uncertainty, frequentists often rely on confidence intervals to provide a range in probability, such as 95% confidence interval. On the other hand, Bayesian methods use credible intervals, which incorporate prior information and provide a probability distribution of the parameter, such as 95% Bayesian credible interval. These differences highlight the distinct philosophies underlying each approach: frequentists view probability as a long-run frequency of events, while Bayesians interpret probability as a degree of belief, updating prior knowledge with new data (Pek & Van Zandt, 2020).

1.6.2 Principal Coordinate Analyses (PCoA)

Principal Coordinate Analysis (PCoA) is a robust technique used for visualizing the proximity and relationships between samples (Torgerson, 1958) based on community composition data. This method relies on matrices derived from an OTU (Operational Taxonomic Unit) table, where rows correspond to OTUs and columns represent different samples, with the values indicating counts of sequences per OTU (Anderson & Willis, 2003). In the case of presence versus absence matrices instead of counts, the OTU tables are converted into a binary matrix that marks the presence or absence of each OTU in the samples. When looking at the phylogenetic relationship between samples, the next step involves constructing a phylogenetic tree, which illustrates the evolutionary relationships among OTUs. This tree helps in identifying distinct and shared branches across samples, which are crucial for understanding sample similarities and differences (Lozupone et al., 2006; Lozupone & Knight, 2005).

Table 1.1 Brief overview of four metrics commonly used in creating Principal Coordinate plots in the field of ecology, with a description and use case for each metric.



Metric	Description	Use Case
Unweighted UniFrac	Phylogenetic distance considering only presence/absence of taxa	Compare community structure without considering abundance
Weighted UniFrac	Phylogenetic distance incorporating relative abundance of taxa	Compare community structure considering both presence and abundance
Jaccard Similarity Index	Similarity based on presence/absence taxon (intersection/union)	Compare binary data or presence-absence data
Bray-Curtis Dissimilarity	Dissimilarity based on abundance and presence (sum of minimum abundances)	Compare community composition considering both abundance and presence

To assess community structure based on the presence or absence of taxa, the Unweighted Unifrac distance matrix is calculated:

$$U_{ij} = \frac{\sum_k \mid l_{ik} - l_{jk} \mid}{\sum_k (l_{ik} + l_{jk})}$$

This method evaluates phylogenetic distances between communities, focusing on taxonomic presence or absence, without accounting for their relative abundances (Lozupone & Knight, 2005). For each pair, the branch lengths unique to each sample and the total branch length of the tree were calculated. The Unweighted UniFrac distance between samples is then calculated to produce a matrix reflecting the dissimilarity between all sample pairs. The Unweighted Unifrac metric is suitable for analyzing community differences that arise from species loss or gain (Lozupone et al., 2006; Lozupone & Knight, 2005).

To account for both the presence/absence and the relative abundances of taxa, the Weighted Unifrac distance matrix is calculated:

$$W_{ij} = \frac{\sum_k w_{ik} \cdot \mid l_{ik} - l_{jk} \mid}{\sum_k w_{ik} \cdot (l_{ik} + l_{jk})}$$

This metric incorporates the abundance of each taxon in the distance calculation, allowing for a more nuanced comparison of community structure (Lozupone et al., 2006). Weighted Unifrac is effective for evaluating differences in community composition and abundance across samples.

The Bray-Curtis dissimilarity metric is used to measure the dissimilarity between samples based on relative abundances:

$$BC_{ij} = \frac{\sum_{k=1}^{S} | x_{ik} - x_{kj} |}{\sum_{k=1}^{S} (x_{ik} - x_{kj})}$$

This non-phylogenetic metric calculates the dissimilarity between samples by comparing the relative abundance of taxa, making it useful for examining differences in community structure without considering phylogenetic relationships (Bray & Curtis, 1957). It ranges from 0 (identical communities) to 1 (completely dissimilar). This metric considers both species presence/absence and abundance, making it sensitive to the relative abundance of species in the communities. The Jaccard dissimilarity index is computed to analyze the community composition based on the presence or absence of taxa, disregarding their relative abundances:

$$J_{ij} = \frac{a}{a + b + c}$$

The Jaccard index measures the proportion of shared taxa between samples and helps understand community differences from a binary perspective (Jaccard, 1901). Lastly, the Shannon Index can be calculated to quantify the diversity of taxa within each sample:

$$H' = -\sum_{i=1}^{S} (p_i \cdot \ln(p_i))$$

This index measures the richness and evenness of species in a community, offering insight into the complexity of each sample’s community structure (Shannon, 1948).

For each distance matrix discussed (Unweighted UniFrac, Weighted UniFrac, Bray-Curtis, and Jaccard) the Principal Coordinate Analysis (PCoA) is performed to visualize the multidimensional relationships between samples in a lower-dimensional space. Each PCoA is created by subjecting the distance or dissimilarity matrix to eigenvalue decomposition. This step transforms the matrix into a set of orthogonal axes (known as principal coordinates) that represent the major dimensions of variation in the data. The principal coordinates corresponding to the largest eigenvalues are used to plot the samples in a lower-dimensional space (typically 2D or 3D). The distances between points on the plots reflect the original dissimilarities between samples. Clustering patterns (indicating similarity) or gradients in the plots may relate to underlying biological or environmental factors.

This study aimed to refine the application of eDNA monitoring to be applied to assessing coral reef spawning activities. While previous studies have confirmed the detection of coral biodiversity in eDNA samples, it remains to be established whether these assays can reliably and consistently detect and quantify the relative abundance of specific coral genera within spawning material. The genus detection and accuracy of relative abundance ratios were verified by comparing expected versus observed results from known mixes and validating the precision of database detections at the genus level using known mix compositions. Additionally, Detection capabilities for identifying corals and other spawners, such as anemones and sponges, within wild spawning slicks were confirmed to distinguish between coral and non-coral-dominated slicks. Limitations in taxonomic resolution were examined by comparing relative abundance data at both the family and genus levels. The utility of Principal Coordinates Analysis (PCoA) was used for describing reef composition. Finally, assay detection capabilities were evaluated by analyzing and comparing the number of unique sequence detections, providing insights into the effectiveness of the assays in capturing reef biodiversity.

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Chapter 2- Manuscript

Author: Bailey Marquardt

Affiliation: University do Algarve

Contact email: bmarquardt@uwalumni.com

Abstract

Monitoring threatened ecosystems is critical for understanding and mitigating the ongoing loss of biodiversity. Coral reefs are among the most vulnerable ecosystems, facing numerous threats including climate change, overfishing, and pollution. Restoration activities, such as coral transplantation and the establishment of marine protected areas, are increasingly employed to restore these ecosystems to their former health. However, the effectiveness and influence on the ecosystem of such restoration efforts are often difficult to assess due to the challenges in monitoring the vast and complex marine environment.

This study focuses on the use of environmental DNA (eDNA) to monitor coral spawning slicks, which are vital for the natural recovery and maintenance of coral populations. Spawning slicks, composed of gametes and zygotes released during synchronized coral spawning events, provide a unique opportunity to assess the reproductive success and species composition of coral communities. While traditional methods for monitoring restored coral populations are labor-intensive and limited in scope, eDNA analysis of slick samples provides a more efficient alternative. By analyzing eDNA from water samples collected during spawning events, we can identify the species present in the slicks, including those that are difficult to detect through visual observations alone.

Utilizing the CoralITS2 and CoralITS2_acro assays, this study demonstrates the potential of eDNA as a powerful tool for monitoring the composition of coral spawning slicks, thereby providing insights into the reproductive dynamics of restored and at-risk coral species. This approach offers a non-invasive, cost-effective, and scalable method for assessing the success of restoration activities and informing conservation strategies. By integrating eDNA monitoring into existing coral reef management practices, we can enhance our ability to track the recovery of these critical ecosystems and better understand the impacts of restoration efforts.

Keywords

Biodiversity, Coral Reefs, eDNA, ITS2, Metabarcoding, Monitoring

2.1 Introduction

The greatest threat to coral reefs is ocean warming driven by anthropogenic greenhouse gas emissions, which have led to widespread impacts on corals (Hoegh-Guldberg et al., 2019). Corals are marine invertebrates that form the foundation of coral reefs by secreting calcium carbonate skeletons, supporting a vast array of marine life, and playing a crucial role in maintaining biodiversity (Maragos et al., 1996) and coastal protection (Pascal et al., 2016). Coral reef ecosystems are highly productive and economically valuable, supporting diverse species, offering protection from floods, and driving local economics through tourism (Arkema et al., 2015; Fezzi et al., 2023; Moberg & Folke, 1999; Reguero et al., 2021). Marine heatwaves threaten global biodiversity as these recent extreme warming events have impacted critical foundation species such as corals, seagrasses, and kelps (Smale et al., 2019). While marine heatwave events do not always result in mass coral mortality, the increasing severity and frequency of these events will result in increasing coral mortality and reef degradation outlined in climate change scenarios by the International Panel on Climate Change (Gadoutsis et al., 2019; Intergovernmental Panel on Climate Change (IPCC), 2023; Maynard et al., 2008; Weis, 2010; Yadav et al., 2023). Marine heatwaves have triggered four mass coral bleaching events on the Great Barrier Reef in just seven years, reducing shallow water coral reefs by as much as 50% (Dietzel et al., 2021; Hughes et al., 2017). Given the widespread habitat degradation, there is a growing acknowledgment that active restoration efforts are essential to ensure the continued viability of natural ecosystems and the services they provide in the medium-term future (Bullock et al., 2011).

Interventions of coral reef restoration include the dispersal of seeding devices with settled recruits (Chamberland et al., 2017; Randall et al., 2021, 2023; Whitman et al., 2024), transplanting fragments from mature adult colonies (Rinkevich, 2005; Whitman et al., 2024; Young et al., 2012), and collection of coral slicks to settle on degraded reef (dela Cruz & Harrison, 2020). Additionally, micro fragmenting is a specific restoration technique that involves breaking larger coral colonies into small pieces to stimulate rapid growth and accelerated wound healing, allowing for faster coverage of degraded reefs and increased genetic diversity, as many genotypes can be propagated and outplanted (Forsman et al., 2006; Page et al., 2018; Rapuano et al., 2023). The effectiveness of these restoration activities is currently evaluated using a combination of ecological monitoring methods, including the survivorship and growth rates of transplanted corals, reef structure, and habitat complexity, though reproductive success is rarely discussed (Boström-Einarsson et al., 2020).

Monitoring and assessing the success of restoration activities currently employs methods similar to traditional coral reef benthic monitoring, such as individual colony tracking and percent cover, with the shared objective of measuring changes in reef conditions over time (Bruno & Selig, 2007; Obura et al., 2019; Wilkinson, 2002, 2008). These methods are typically labor intensive and involve divers surveying areas with photographic equipment or noting live results during surveying periods. Reproductive success is difficult to assess, as it can take a range of years (three to eight years or more) for a coral to reach sexual maturation (Cruz & Harrison, 2017; Kojis & Quinn, 1985), and the factors that determine the onset of sexual maturation in corals and the extent to which this onset can be phenotypically plastic remain largely unknown (Randall et al., 2020). Consistent, comparable, and quantitative data are required to quantify the changes resulting from restoration activities, yet these traditional methods often focus on the individuals outplanted, neglecting the broader reproductive contributions of the restored population to the ecosystem.

Scleractinia corals, which undergo a biphasic life cycle composed of a dominant sessile, benthic polyp phase, and a motile planula larval phase (Harrison, 1985, 2011; Harrison & Wallace, 1990; Harrison et al., 1984; Willis et al., 1985), offer a more holistic perspective on restoration success when their reproductive output is considered. Once corals reach adequate size and age, they may engage in synchronized spawning events, releasing billions of gametes per hectare of reef (Álvarez-Noriega et al., 2016; Harrison, 2011; R. L. Harrison et al., 1984; Howells et al., 2016; Willis et al., 1985). These mass spawning events generate species-rich surface slicks that reflect the reproductive contributions of the local coral population (Butler, 1980; Oliver & Willis, 1987). Importantly, the composition and viability of these slicks are directly influenced by the abundance and fecundity of the adult corals on the reef (Hughes et al., 2000; Randall et al., 2020), making them a critical indicator of restoration success at the community level. The presence of dense and diverse slicks signals that multiple coral species are contributing to the reproductive output, serving as a broader community-level indicator of restoration efficacy (Boström-Einarsson et al., 2020). Furthermore, these slicks can provide insights into larval dispersal potential, which is crucial for the successful recruitment of corals to both restored and nearby reefs, thereby enhancing natural recovery and resilience (Lukoschek et al., 2013).

Environmental DNA (eDNA) refers to genetic material obtained directly from environmental samples and is a method that has shown great promise in investigating marine ecosystems, where metabarcoding techniques are being used for biodiversity assessments (Alexander et al., 2020; Cilleros et al., 2019; Dugal et al., 2022; Everett & Park, 2018; Thomsen et al., 2012). Metabarcoding studies to investigate coral biodiversity have successfully leveraged the CoralITS2 and CoralITS2_acro primers, which target highly conserved regions of the mitochondrial genome for taxon specificity but sufficient variation for genus distinction (Alexander et al., 2020). Leveraging eDNA methods offers significant potential by providing a non-invasive, efficient, and scalable approach to tracking species diversity and abundance across large spatial and temporal scales.

Effective coral reef management depends critically on the precision and thoroughness of biodiversity data collection, particularly regarding species richness and distribution (Deiner et al., 2017; Rees et al., 2014). Moreover, monitoring spatial and temporal variations in coral diversity is essential for informing managers about shifts in reef community structure (Ryan et al., 2014). Long-term coral reef monitoring programs play a key role in enabling scientists and managers to assess reef health and enhance our understanding of local-scale trends (Lasagna et al., 2014).

This study aimed to refine the application of eDNA monitoring to be applied to assessing coral reef spawning activities. While previous studies have confirmed the detection of coral biodiversity in eDNA samples, it remains to be established whether these assays can reliably and consistently detect and quantify the relative abundance of specific coral genera within spawning material. The genus detection and accuracy of relative abundance ratios were verified by comparing expected versus observed results from known mixes and validating the precision of database detections at the genus level using known mix compositions. Additionally, detection capabilities for identifying corals and other spawners, such as anemones and sponges, within wild spawning slicks were confirmed to distinguish between coral and non-coral-dominated slicks. Limitations in taxonomic resolution were examined by comparing relative abundance data at both the family and genus levels. The utility of Principal Coordinates Analysis (PCoA) was used for describing reef composition. Finally, assay detection capabilities were evaluated by analyzing and comparing the number of unique sequence detections, providing insights into the effectiveness of the assays in capturing reef biodiversity.

2.2 Materials and Methods

2.2.1) Study Site and Wild Sample Collection

Four reef sites along the Great Barrier Reef were sampled opportunistically during the 2018 and 2021 spawning seasons (Figure 2.1). Coral spawning slick material was collected from Backnumbers Reef (-18.5087, 147.1529), Keeper Reef (-18.7492, 147.2656), Lizard Island (-14.6802, 145.4466), and the Whitsundays (-20.1705, 148.9225) (Table 2.1). Coral spawning was visually confirmed by the presence of spawning slicks at the surface of each reef. Slick material was scooped from the water surface and 3-5 mL of the slick was transferred with sterile transfer pipettes to 15 mL sterile sample tubes. Samples were fixed in 100% ethanol and stored at room temperature until processing for DNA extraction.

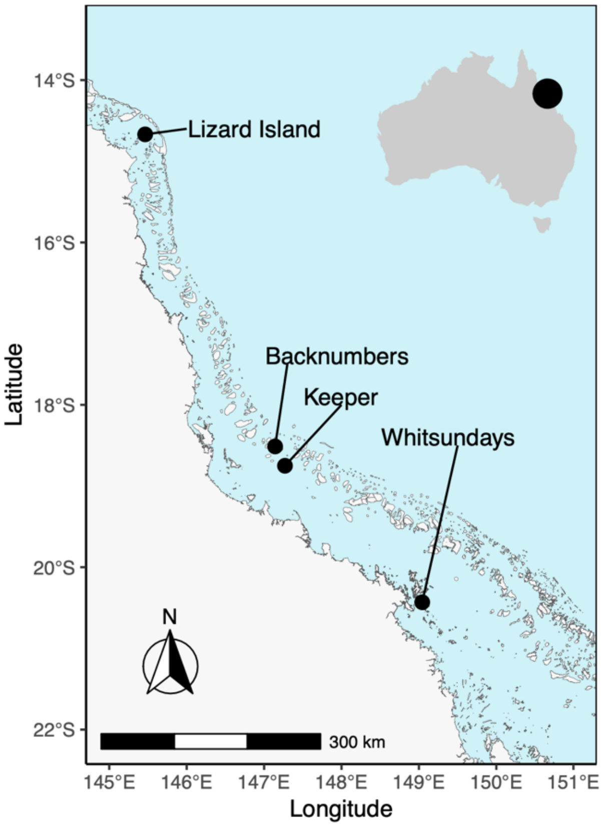
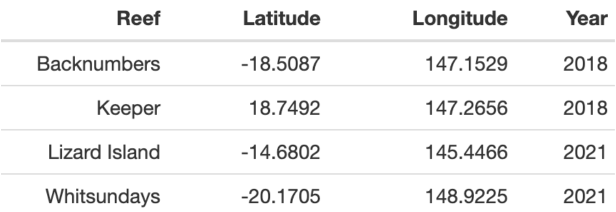


Figure 2.1 Wild slick sample collection sites along the Great Barrier Reef, Australia.

2.2.2) Reference Material (Known Mix) Creation

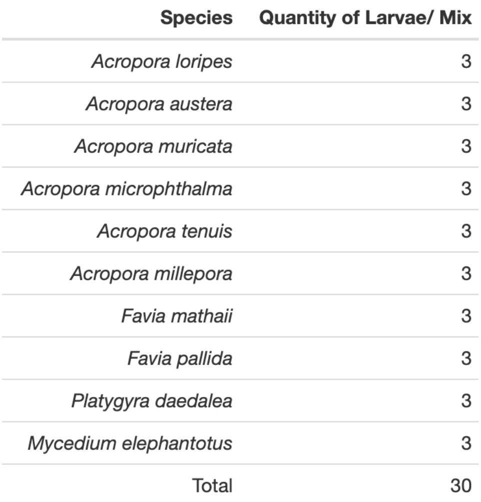
To assess the detection capabilities of eDNA assays, larvae from ten species from ten bulk crosses of coral larvae made from known parents previously identified in the field and kept in pure larvae cultures. These larvae were pooled to create what we will refer to as a known mix (Table 2.2). Larvae from each species were sampled during the 2018 spawning season at the National Sea Simulator Facility (SeaSIM) at the Australian Institute of Marine Science (AIMS) in Townsville, Australia. Five replicate known mixes were created, and each mix contained three larvae of each species for a total of 30 larvae in each mix. Samples were fixed in 100% ethanol and stored at room temperature until further processing for DNA extraction. The sampling equipment was sterilized with a fresh water and household bleach (10%) solution, and fully dried between use.

Table 2.1 Wild slick sample collection data



Reef	Latitude	Longitude	Year
Backnumbers	-18.5087	147.1529	2018
Keeper	18.7492	147.2656	2018
Lizard Island	-14.6802	145.4466	2021
Whitsundays	-20.1705	148.9225	2021

Table 2.2 List of coral species and quantities used to create the known mixes

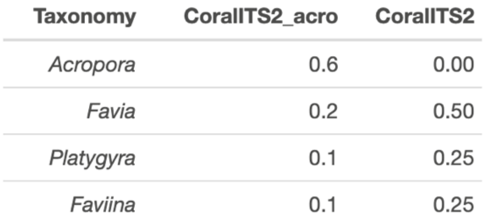


Species	Quantity of Larvae/ Mix
Acropora loripes	3
Acropora austera	3
Acropora muricata	3
Acropora microphthalma	3
Acropora tenuis	3
Acropora millepora	3
Favia mathaii	3
Favia pallida	3
Platygyra daedalea	3
Mycedium elephantotus	3
Total	30

2.2.3) Expected Known Mix Ratios

The CoralITS2 and CoralITS2_acro assays are expected to provide genus-level resolution for taxonomic identification (Alexander et al., 2020; Dugal et al., 2022). Based on environmental monitoring with the same primers, CoralITS2 is anticipated to detect the genera *Favia* and Platygyra, and possibly *Mycedium* at the previously assigned family level (Faviina), but will not detect *Acropora* species (Table 2.3, S2). It is expected that the CoralITS2_acro assay will detect *Acropora, Favia,* and *Mycedium*, at the previously assigned sub-order level (Faviina) due to database resolution (Table 2.3, S2).

Table 2.3 Expected abundance ratios of known mix extraction per assay type



Taxonomy	CoralITS2_acro	CoralITS2
Acropora	0.6	0.00
Favia	0.2	0.50
Platygyra	0.1	0.25
Faviina	0.1	0.25

2.2.4) eDNA Extraction, Metabarcoding, and Library Preparation

All extraction, quantification, and sequencing of eDNA were completed in dedicated clean laboratories wearing nitrile gloves to reduce the risk of non-sample DNA contamination. DNA was extracted following a modified DNA extraction protocol specifically for pools of larvae, which includes a 2.5-3-hour incubation in buffer solution and RNase (Wilson et al., 2002).

Known mix extracts and eDNA samples underwent two metabarcoding assays (CoralITS2 and CoralITS2_acro; Table S1) which target the ITS2 gene (Alexander et al., 2020a; Brian et al., 2019; West et al., 2022). Amplicons were approximately 419 bp for CoralITS2 and 445 bp for CoralITS2_acro. The ITS2 region of Acropora (the dominant coral genus in the known mix and study locations) is shorter than the ITS2 regions of other coral species. This shorter region in the CoralITS2 assay impedes efficient primer binding, thus hindering the detection of the Acropora genus, therefore the CoralITS2_acro assay, which includes a modified reverse primer, was chosen for this study.

Polymerase chain reaction (PCR), library prep, and Illumina MiSeq sequencing were completed at the Australian Genome Research Facility (Sydney, Australia). Each PCR reaction mixture (25 µL) contained 10x PCR Gold buffer, 50 nM $MgCl_2$, 25 nM dNTPs, 5x SYBR Green, 1 U AmpliTaq PCR buffer, 10 µM of forward and reverse primer, 20 µM of forward and reverse tags, and DNA template. The thermal cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds, with a final extension of 72°C for 10 min. Separate libraries were created for the CoralITS2 samples and the CoralITS2_acro samples. The final libraries were size-selected to a range of 160–550 bp (for CoralITS2) and 175–600 bp (for CoralITS2_acro). The cleaned libraries were then quantified and sequenced across MiSeq 500-cycle V2 chemistry for paired-end sequencing runs (Illumina, USA).

2.2.5) Filtering and Quality Control of Illumina Reads

For each sequencing library, qiime2 (v. 2024.5; Bolyen et al., 2019) was used to demultiplex reads based on unique barcode sequences to separate reads according to the sample. Barcodes were then removed to retain only the relevant biological sequences. DADA2 (Callahan et al., 2016) was used to trim reads, remove phiX rear, dereplicate, merge reads, remove chimeras, and cluster into Amplicon Sequence Variants (ASVs). ASVs are exact DNA sequences that represent distinct taxa within a sample. Unlike Operational Taxonomic Units (OTUs), which group sequences based on a similarity threshold, ASVs maintain the highest resolution, allowing for the precise distinction between closely related organisms (Callahan et al., 2016). The LULU algorithm was then used to remove potential sequencing artifacts and generate ASV datasets based on sequence similarity and ecological co-occurrence patterns. The LULU algorithm merges "daughter" ASVs with consistently co-occurring, more abundant "parent" ASVs (Frøslev et al., 2017). Taxonomy was assigned with the MEGAN (MEtaGenome ANalyzer) (Huson et al., 2007). ASVs were compared against the database of known reads, GENBANK, using BLAST, which produced a list of potential matches (hits) for each read. Results were analyzed using the Lowest Common Ancestor (LCA) algorithm to assign taxa in MEGAN. For each read, the LCA algorithm considers all the taxa that the read aligns within the reference database. The algorithm then scores these matches and applies filtering criteria (such as minimum alignment score and percent identity) to reduce the number of potential matches. Among the filtered matches, the lowest taxonomic level that is common to all the potential matches is identified. This common ancestor is assigned as the taxonomic classification for that read. The LCA parameter top percent was adjusted to create the best taxonomic matches based on the known mix. Sequences with no matches, or taxonomic assignment only at the domain level were discarded.

2.2.6) Statistical and Ecological Analysis

After taxonomic assignment, data from the CoralITS2 and CoralITS2_acro outputs were compared among the known mixes. Average relative abundance and the standard deviation were calculated to visualize the variation in detections. The expected versus the observed outcomes of the average relative abundance were explored using a hierarchical model in a Bayesian framework (Gelman, 2003). Specifically, a beta-binomial model with weakly informative priors was constructed:

$$\begin{aligned} Y|\pi &\sim Bin(n, \pi) \\ \pi &\sim Beta(\alpha, \beta) \\ \pi|(Y = y) &\sim Beta(\alpha + y, \beta + n - y) \\ \beta_0 &\sim t(3, 0, 2) \\ \sigma &\sim t(3, 0, 1) \\ \phi &\sim \Gamma(0.01, 0.01) \end{aligned}$$

The Bayesian model included three chains, each 5,000 interactions, thinned by a factor of ten, with the first 1,000 iterations used for warmup and excluded from the analysis. The model demonstrated good mixing and convergence (all $\hat{\alpha} < 1.01$) on a stable posterior (Supp#). Validation was performed using simulated residuals with the DHARMa package (Hartig, 2022; R Foundation for Statistical Computing, 2024). All statistical analyses were performed in the R Statistical and Graphical Environment (version 4.4.1; R Core Team, 2024) via the brms package (Bürkner, 2017). Post-hoc comparisons between observed and expected values were made based on the full posteriors before being summarized using draw-level chi-squared values, medians, and highest probability intervals.

Four principal coordinate analyses (PCoAs) were created to visualize sample proximity. Coral community composition data included counts of various OTUs across different reef spawning slick samples. To calculate the various matrices of each PCoA, an OTU table where rows represent OTUs, and columns represent samples, containing counts of sequences per OUT was utilized to calculate various matrices. In the case of presence versus absence matrices, the table was converted into a binary matrix indicating the presence or absence of each. A phylogenetic tree was constructed that depicts the evolutionary relationships among OTUs and was used to identify unique and shared branches for each pair of samples (Bolyen et al., 2019).

For each distance matrix calculated (Unweighted UniFrac, Weighted UniFrac, Bray-Curtis, and Jaccard) the Principal Coordinate Analysis (PcoA) was performed to visualize the multidimensional relationships between samples in a lower-dimensional space. Each PcoA was created using the cmdscale function in R (R Core Team, 2024) and visualized with Rstudio (v2024.4.2.764; Posit Team, 2024). For each distance matrix, the function was applied to extract the principal coordinates, which were then plotted to visualize the clustering patterns and relationships among samples. The resulting ordination plots were used to interpret the overall community structure and differences among the samples based on the distance metrics and the Shannon Index.

2.3 Results

2.3.1) Reference Material (Known Mixes) Sequencing Results

Metabarcoding of the mixes containing 3 larvae of each 10 known species yielded a total of 95,172 and 72,919 demultiplexed sequences, with an average of 31,724 and 24,306 reads per sample for the CoralITS2_acro and CoralITS2 assays respectively. All known mixes were amplified during PCR. The negative PCR control did not amplify any DNA in the CoralITS2_acro assay but did amplify DNA in the CoralITS2 assay, thus the negative control for the CoralITS2 assay was sequenced. After LULU, there were an average of 54 and 8 ASVs per sample from the CoralITS2_acro and CoralITS2, respectively. The PCR product from the negative control of the CoralITS2 assay was mapped to two ASVs that were not present in any other sample (Table S3).

The CoralITS2_acro assay resulted in an average of 65 ASVs per sample, five of which showed no known taxonomic match. The previous suborder *Faviina* (of the *Favia,* and *Mycedium* genera in the known mix), and the genus *Platygyra* were the most resolved taxonomic assignments for their respective ASVs. Due to the limited resolution of *Faviina*, for the expected ratios for the assay, we categorize *Favia* and *Mycedium* into one expected group to make up approximately 30% of the relative abundance.

When reviewing the genus level where possible, the average relative abundance in each known mix is comparable to what is expected given the known mix composition (Figure 2.2, Figure 2.3). The expected relative abundance per taxonomy was as follows: *Acropora* (60%), *Faviina* (30%), *Platygyra* (10%), and the observed values were: *Acropora* (58.5%), *Faviina* (32.8%), *Platygyra* (8.7%) (Table 2.4, Figure 2.3). There was significant variation in *Acropora* and *Faviina* relative abundance between replicates resulting in a standard deviation of 18.9% and 15.5% respectively. *Platygyra* showed average values close to the expected relative abundance levels, and exhibited more consistency between samples, resulting in a low standard deviation of 3.3%.

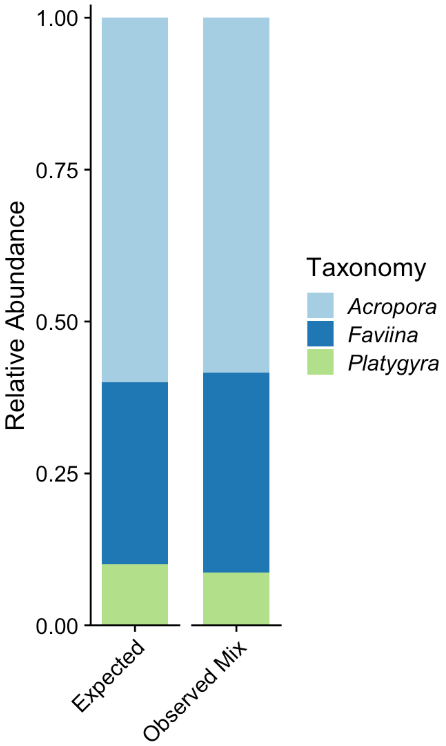


Figure 2.2. Bar plot showing expected versus observed relative abundance of taxa for the known mixes of the CoralITS2_acro assay.

Table 2.4. Average relative abundance and standard deviation for each detected taxonomy per type of expected ratios versus the observed mix for the CoralITS2_acro assay.

Type	**Taxonomy**	**Average Relative Abundance**	**sd**
Observed Mix	Acropora	0.585	0.189
Observed Mix	Faviina	0.328	0.155
Observed Mix	Platygyra	0.087	0.033
Expected	Acropora	0.600	NA
Expected	Faviina	0.300	NA
Expected	Platygyra	0.100	NA

Exploring the data with a Bayesian beta-binomial model revealed that observed relative abundance values do not deviate from the expected values (median deviation = 0.065, 95% CI [-0.27 – 0.5]; Figure 2.3). There is no evidence that the observed relative abundance of each taxon deviates from the expected values (x < 0 = 0.329, x > 0 = 0.671). Thus, observed data align well with the expected values, suggesting that the variations observed are consistent with random fluctuation rather than a systematic effect.

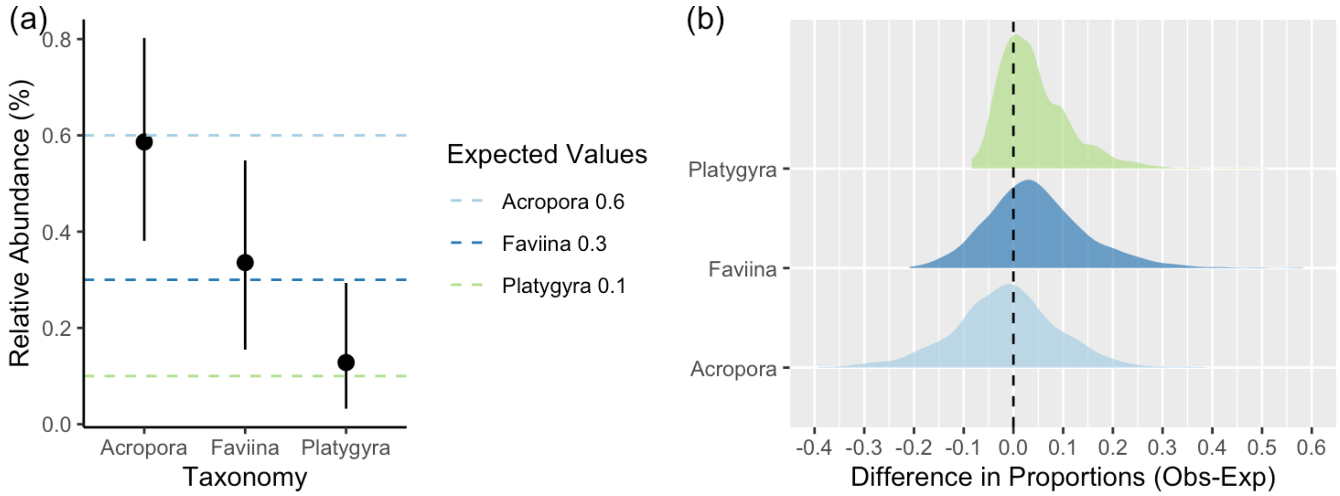


Figure 2.3 Forest plots for CoralITS2_acro assay beta-binomial model for relative abundance of the known mixes. (a) Model estimation of relative abundance per taxonomy. Dashed horizontal lines indicate expected values per taxonomy. Points represent median value, and solid lines represent the 95% credibility intervals. (b) Model effectiveness assessment evaluating the difference in proportions of observed versus expected relative abundance. The dashed vertical line represents the expected difference of 0. The horizontal line shows the difference between observed and expected values per taxa for all draws in the model.

The CoralITS2 assay resulted in 10 ASV matches; 8 that mapped to known taxonomy in the sample, 1 that mapped to a genus not present in the sample (*Physophyllia)*, and 1 ASV that resulted in no known taxonomic match. The previous suborder *Faviina* (of the *Favia, Mycedium, and Platygyra* in the known mix), the family *Merulinidae (current classification of Platygyra),* and the genus *Favia* were the most resolved taxonomic assignment for their respective ASVs. Given that the *Favia* genera and *Merulinidae* (*Platygyra* family) are detected*,* the Faviina detections may represent *Mycedium* in the known mix because the taxonomy in the database could still reference this older taxonomic definition*.* Thus, the expected relative abundance ratios are unaffected, but the detected taxonomy resolution is adjusted (Table 2.5).

When reviewing the genus level, the average relative abundance in each known mix is comparable to what is expected given the known mix composition (Figure 2.4, Table 2.5). The expected relative abundance per taxonomy was as follows: *Favia* (50%), *Faviina* (25%), *Platygyra* (25%), and the observed values were: *Favia* (48.9%), *Faviina* (19.3%), *Merulinidae* (25.9%), and *Phosphyllia* (5.9%) (Table 2.5). There was no significant variation in relative abundance between replicates resulting in a standard deviation of *Favia* (6.5%), *Faviina* (9.4%), *Merulinidae* (6.7%), and *Phosphyllia* (2.7%) (Table 2.5).

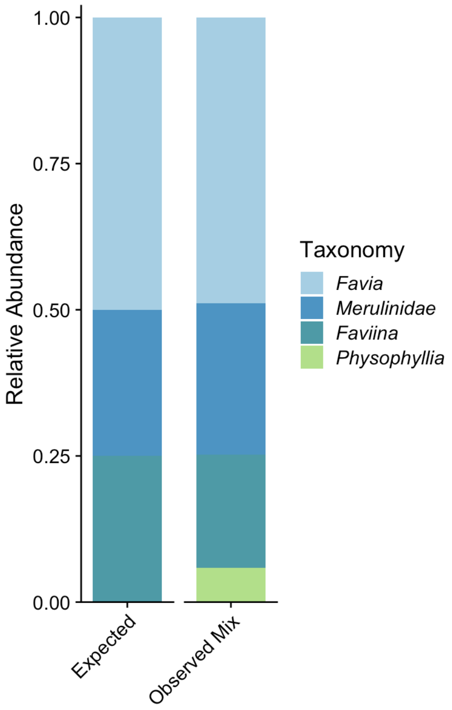


Figure 2.4. Bar plot showing expected versus observed relative abundance of taxa for the known mixes and the CoralITS2 assay.

Table 2.5. Average relative abundance and standard deviation for each detected taxonomy per type of expected ratios versus the observed mix for the CoralITS2 assay.

Type	Taxonomy	Average Relative Abundance	sd
Observed Mix	Favia	0.489	0.065
Observed Mix	Faviina	0.193	0.094
Observed Mix	Merulinidae	0.259	0.067
Observed Mix	Physophyllia	0.059	0.027
Expected	Favia	0.500	NA
Expected	Faviina	0.250	NA
Expected	Merulinidae	0.250	NA

Observed relative abundance values align well with the expected values (median deviation = 0.0622, 95% CI [-0.32 – 0.47]; Figure 2.5), regardless of the detection that was not expected in the known mix (*Physophilla)*. There is no evidence that the observed relative abundance of each taxon deviates from the expected values ($x < 0 = 0.351$, $x > 0 = 0.649$). These results suggest that the observed data align well with the expected values and that the variations observed are consistent with database quality, DNA quality, and random fluctuations versus a systemic effect.

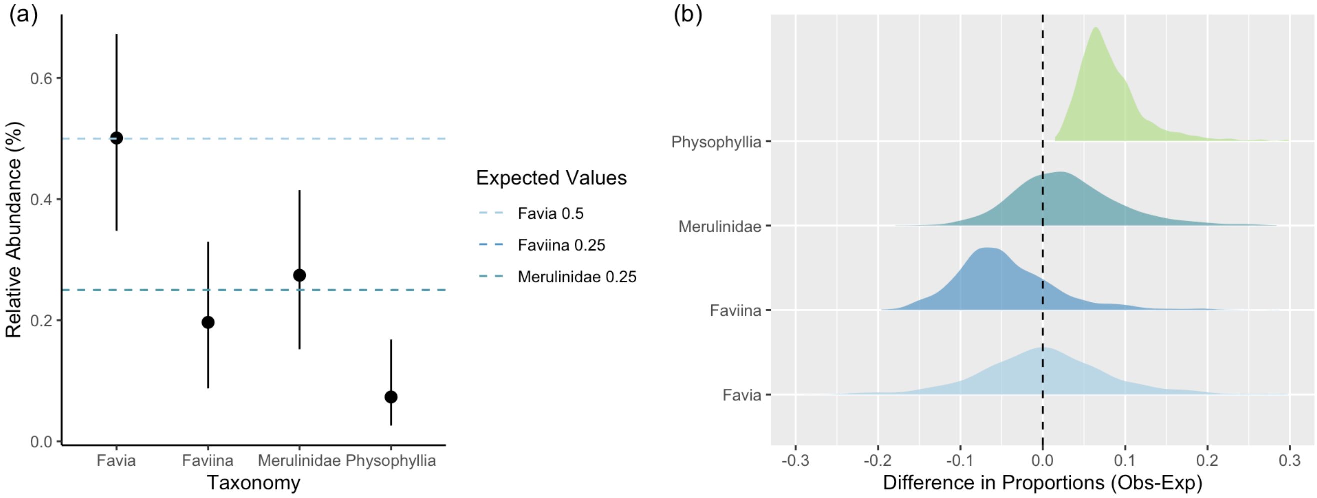


Figure 2.5 Forest plots for CoralITS2 assay beta-binomial model for relative abundance of the known mixes. (a) Model estimation of relative abundance per taxonomy. Dashed horizontal lines indicate expected values per taxonomy. Points represent median value, and solid lines represent the 95% credibility intervals. (b) Model effectiveness assessment evaluating the difference in proportions of observed versus expected relative abundance. The dashed vertical line represents the expected difference of 0. The horizontal line shows the full posteriors as differences between observed and expected values per taxa for all draws in the model.

2.3.2) Wild Slick Sequencing Results

Metabarcoding of the wild samples yielded a total of 632,909 and 382,586 demultiplexed sequences, with an average of 37,230 and 23,911 reads per sample for the CoralITS2_acro and CoralITS2 assays respectively. All but one sample was amplified during PCR using ITS2 and ITS2_acro. One wild slick sample from Whitsundays_3 failed to amplify during PCR and was not sequenced, thus there are only two samples for this reef site.

The CoralITS2_acro assay resulted in an average of 39 ASVs per sample, five of which showed no known taxonomic match. S20_Whitsundays_3 resulted in 293 OTUs that mapped to a single ASV, which had no known taxonomic match. Thus, S20_Whitsundays_3 was removed from further analysis and there are only two samples for this reef site in the CoralITS2_acro results.

When taxonomy is resolved from the CoralITS2_acro assay to the family level the complete relative abundance diversity is captured, as this broad taxonomic resolution fully represents the total diversity in the samples (Figure 2.6, Table S4). *Acroporidae* is the predominant coral family in most samples, with nearly 100% relative abundance in Keeper and Lizard_Cooper samples. The next most detected taxa is *Faviina*, a previous suborder, and parent of taxa *Merulinidae* and *Mussidae,* which is abundant in the Backnumbers, Lizard, and Whitsundays samples. *Poritidae, Merulinidae, Musside,* and *Zoantharia* are generally absent or present in very low quantities across most samples (Figure 2.6, Table S4). All assigned taxonomic detections with the CoralITS2_acro assay are corals.

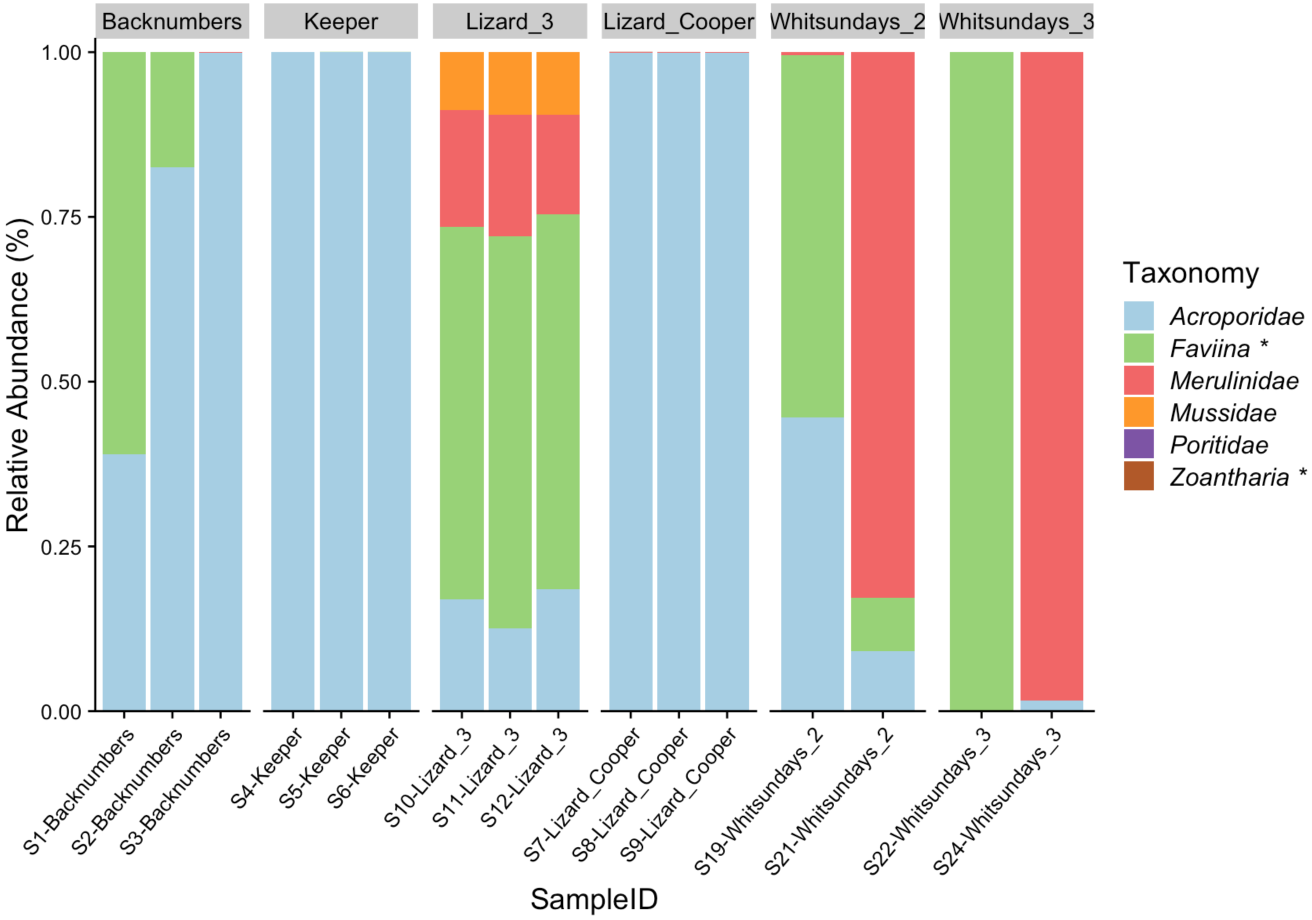


Figure 2.6 Bar plot showing relative abundance results of the CoralITS2_acro assay on the wild slick samples, resolved to the family level where possible. Taxonomic groups that are not resolved to the family level are noted with an (*). *Faviina* is a previous sub-order of coral taxonomic classification.

As the taxonomic resolution is resolved to the genus level, and ASVs that cannot be classified at the genus level are labeled as "NA", the explained diversity shows a substantial reduction. This reduction varies widely from 0% to 100% within samples, with an average decrease of 31% (Figure 2.7, S5). Nearly all the NAs are due to the unresolved taxa within the *Faviina* and *Merulinidae* families. *Acropora* is consistently the most abundant coral genus detected across almost all samples, the exception being Whitsundays_3. These findings also show *Platygyra* as the second most abundant coral that is resolved to the genus level of the CoralITS2_acro assay detections.

The relative abundance shows a significant distribution of taxa across reef sites (Figure 2.6, 2.7). *Acropora* emerged as the most prevalent at Backnumbers, Keeper, and Lizard_Cooper, with significant variation at Backnumbers with a standard deviation of 31.6%, and consistent ratios across replicates for the later sites (sd < 0.01%) (Table S5).

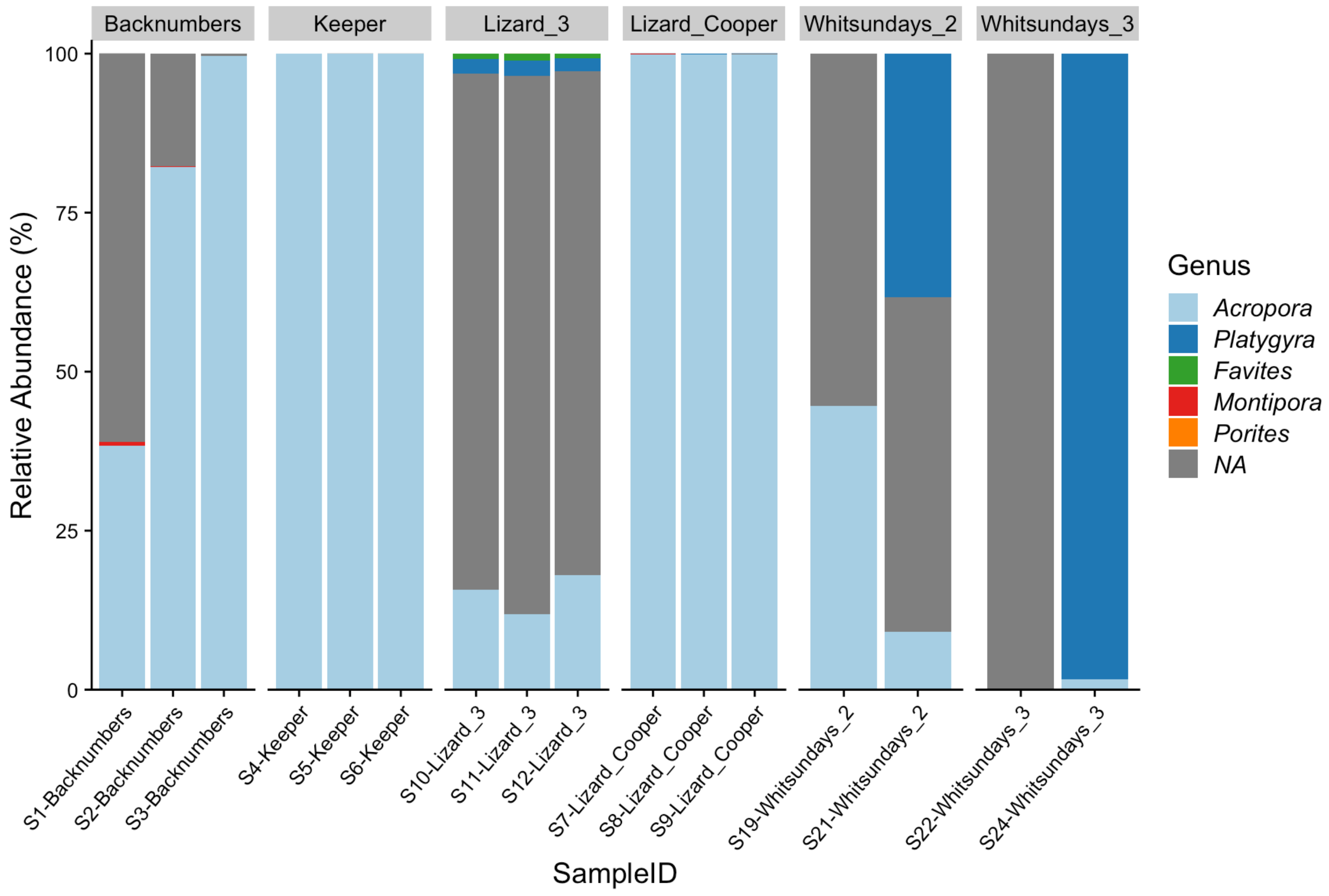


Figure 2.7 Bar plot showing relative abundance results of the CoralITS2_acro assay on the wild slick samples, resolved to the genus level where possible. Grey bars represent ASVs that were not resolvable to a genus level.

The Lizard_3 spawning slick was the most diverse when considering the number of taxa present in the slick and shows consistent relative abundance across replicates as the standard deviation was highest in *Acropora* (3.1%) but generally low among other detections (<1%) (Table S4, S5). The two Whitsundays sites are very diverse in taxa detections across replicates. Whitsundays_2 samples detect similar taxa yet at significantly different abundances resulting in large standard deviations in *Acropora* (25.1%)*, Faviina* (33.1%)*,* and *Merulinidae* (31.1%) (Figure 2.7; Table S4, S5). Additionally, only one replicate detected *Platygyra*, with a substantial relative abundance of 27.1% (Figure 2.7; Table S5). Due to the variation between samples from the Whitsundays, potentially due to processing errors, the average relative abundance for these sites provides questionable insight into the spawning slick composition.

The CoralITS2 assay resulted in 77 unique ASVs, with an average of 9 ASVs per sample, 16 of which showed no known taxonomic match. S23_Whitsundays_3 resulted in 1 OTU that mapped to a single ASV, while S23_Whitsundays_3 was included in relative abundance detection analysis it was removed from the PCoA due to a lack of data that made it incomparable with other samples. S6_Keeper failed to sequence, resulting in 0 OTUs, thus this sample was removed from further analysis and there are only two samples for the Keeper reef site in the CoralITS2 results.

When taxonomy is resolved from the CoralITS2 assay to the family level the complete relative abundance diversity is captured, as this broad taxonomic resolution fully represents the total diversity in the samples, aside from *Faviina* and *Scleractinia* (Figure 2.8). *Mussidae, Merulinidae, and Alcoyoniidae* are the predominant coral family in most samples. Of the 11 detected families, 3 are not corals. *Aplysinidae* and *Clionaidae* are families of sponges and are detected in very small quantities. However, *Actiniidae* is a family of sea anemones and was detected in large abundances in Keeper samples (78% and 82%)(Table S6). This suggests that the collected slick material at Keeper Reef was largely a result of spawning sea anemones.

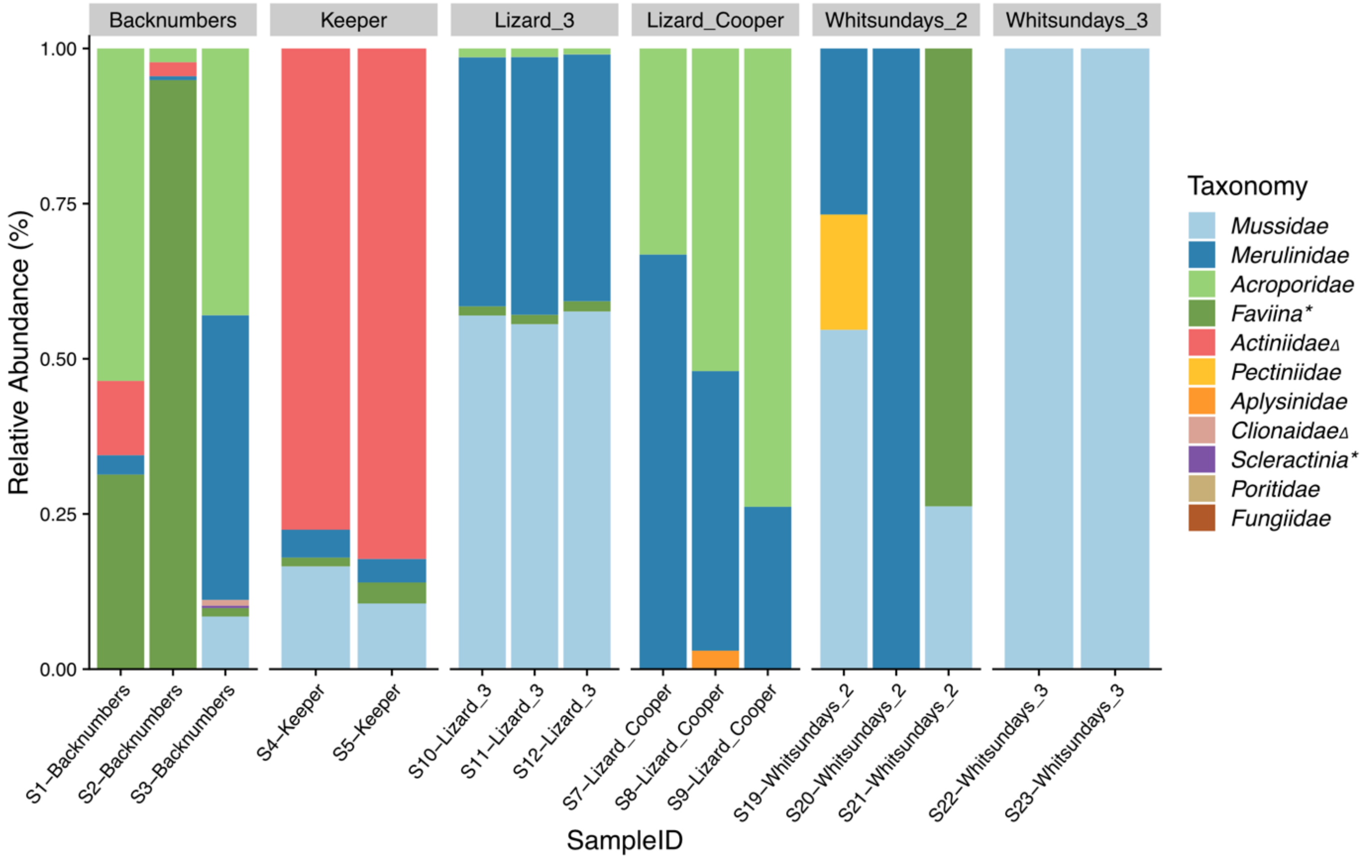


Figure 2.8 Bar plot showing relative abundance results of the CoralITS2 assay on the wild slick samples, resolved to the family level where possible. Taxonomic groups that are not resolved to the family level are noted with an *. *Faviina* is a previous sub-order of coral taxonomic classification. **family** notes detections that are not coral.

As the taxonomic resolution is resolved to the genus level, and ASVs that cannot be classified at the genus level are once again labeled as "NA", there is a reduction in the overall explained diversity. This reduction varies widely from 0% to 100% within samples, with an average decrease of 37% (Figure 2.9, Table S7). Similar to the CoralITS2_acro assay, this reduction in explained diversity is largely due to the resolution of the *Merulinidae* and *Faviina* groups.

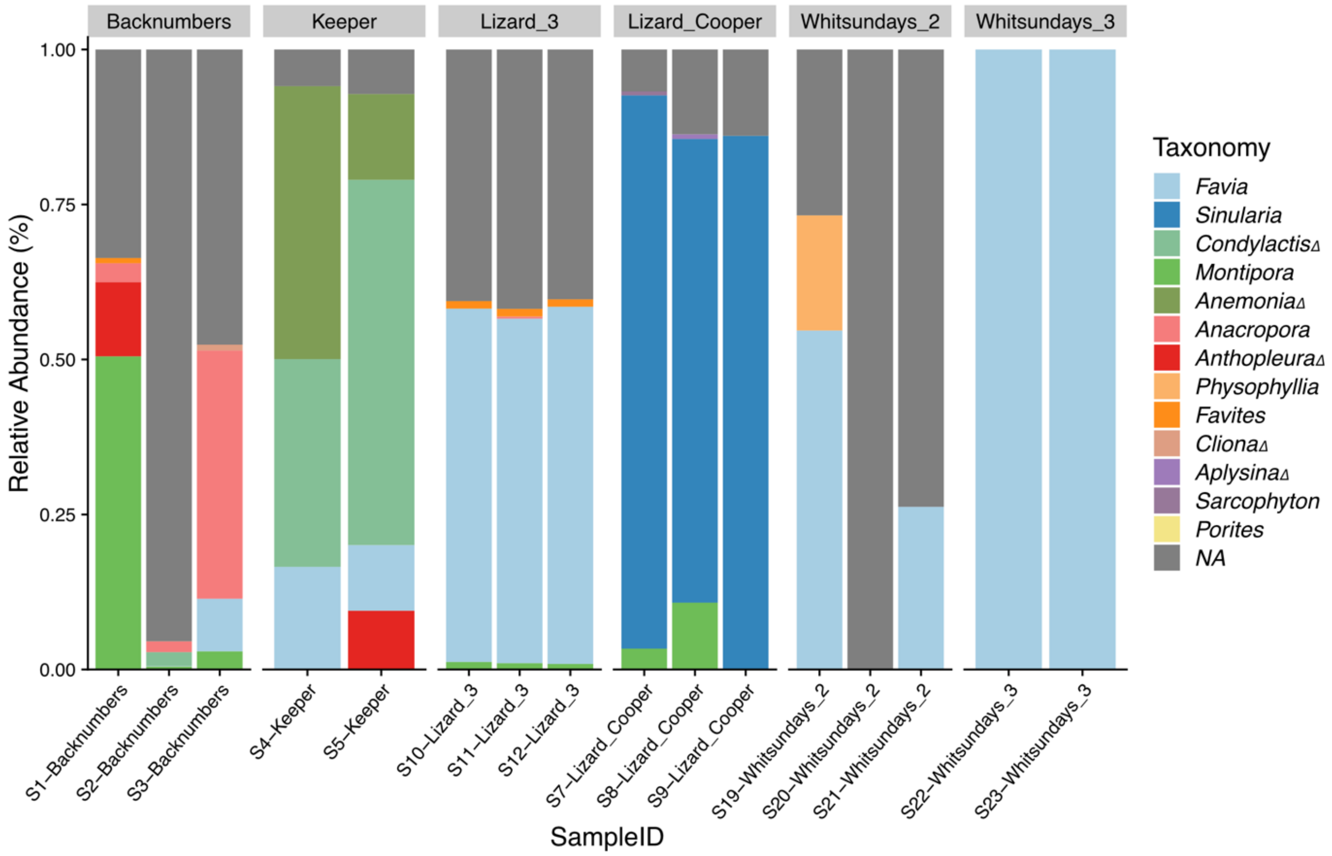


Figure 2.9 Bar plot showing relative abundance results of the CoralITS2 assay on the wild slick samples, resolved to the genus level where possible. Grey bars (NA) represent ASVs that were not resolvable to a genus level. **genus** notes detections that are not coral.

Similar to the CoralITS2_acro results, the relative abundance shows a significant distribution of taxa across reef sites (Figure 2.8, 2.9). *Mussidae, Merulinidae, Acroporidae*, and *Faviina* were the most prevalently detected taxonomic groups across reef sites. The Backnumbers spawning slick was the most diverse when considering the number of taxa present in the slick, however inconsistent relative abundance across replicates is observed as the standard deviations for *Montipora* and *Anacropora* are 28% and 22%, respectively (Table S7). Lizard_3 and Lizard_Cooper sites are diverse in taxa detected and detections are relatively consistent between replicates (sd 1-3%). Whitsundays_2 samples detect a wide range of taxa. Among the commonly detected families, Merulinidae and Mussidae show significant variation in relative abundance, with standard deviations of 52% and 27%, respectively (Table S6). While there was a single ASV detected in S23-Whitsundays_3, both samples of this reef site detected the same single genus, *Favia*, at 100% (Table S7).

2.3.3) Principal Coordinates Analysis (PCoA)

All distance plots used in this study show samples clustered based on similarities. Each distance metric provides unique insights into community composition, with clusters indicating samples that share a high similarity, while sample separation suggests differences. Limited or no clustering implies that the communities are generally similar across locations, with minimal differentiation based on the conditions of each site. As there is a cross-over between detected families and genera, it is expected there may be similar trends observed between CoralITS2 and CoralITS2_acro plots.

In the unweighted UniFrac distance plot, samples are visualized based on their phylogenetic composition. In both the CoralITS2_acro and CoralITS2 plots, clustering is observed within reef sites, reflecting sample similarity. Notably, in the CoralITS2_acro plot, there is a clustering of the Backnumbers and Lizard_3 sites, Keeper, and both Whitsundays sites, with Lizard_Cooper as an outlier. However, in the CoralITS2 plot, the clusters shift to show more even spacing between reefs, with Keeper and Lizard_Cooper grouped more closely. This variation in clustering groups between plots suggests environmental factors may influence the connectivity of corals in the same locations in different ways, driving various phylogenetic connectivity within reef groups.

Unlike the unweighted UniFrac plot, Weighted UniFrac distance accounts for both phylogenetic composition and relative abundance. Clustering indicated similarity in both community composition and abundance. Clustering of samples indicates similarity in both composition and abundance, which is noted within the replicates of reef sites, and additionally Lizard_3, and the Whitsundays sites in the CoralITS2_acro assay. Outliers such as the Lizard_Cooper, Backnumbers, and Keeper reef sites represent unique community profiles with distinctive abundance patterns. There is a lack of clustering among the Coral_ITS2 samples, suggesting the groups are relatively similar to one another. This suggests there is low variability within the samples, or that the corals identified with the Coral_ITS2 assay show a baseline similarity in community structure that hasn't substantially deviated under each location given environmental conditions.

In the Bray-Curtis dissimilarity plot, samples are visualized based on their community composition and abundance. Samples that cluster together have similar taxonomic compositions and abundance, while those farther apart are more dissimilar. In the CoralITS2_acro each reef site shows general clustering, additionally, the Backnumbers Keeper and Lizard_Cooper reef sites are more like one another than the Lizard_3. Additionally, the Whitsundays_2 and Whitsundays_3 replicates are farther away from each other, suggesting dissimilarity between replicates. These results align with the relative abundance patterns.

The Jaccard distance plot shows clustering among CoralITS2 and CoralITS2_acro samples, indicating that certain groups of samples exhibit high similarity in their presence/absence patterns of identified taxonomy. Again, the Whitsundays samples are more diverged from one another. The Shannon index value remains identical for each sample across all plots, as this value is reflective of each sample's diversity. The larger the bubble, the greater the Shannon index, which reflects higher diversity. For samples from Keeper, Lizard_Cooper, the Shannon value is greater for more diverse samples with a more even distribution of taxa. Lower values indicate less diversity and potentially more dominance by a few species, as seen in both Whitsundays samples.

Across all distance plots, including unweighted UniFrac, weighted UniFrac, Bray-Curtis dissimilarity, and Jaccard distance, similar clustering patterns within reef sites are observed. Each plot reveals consistent groupings of reef sites, with certain locations showing tight clustering while others appear more dispersed. This consistency suggests that the underlying patterns of community composition and similarity are robust across different analytical methods, highlighting the stability of the observed trends.

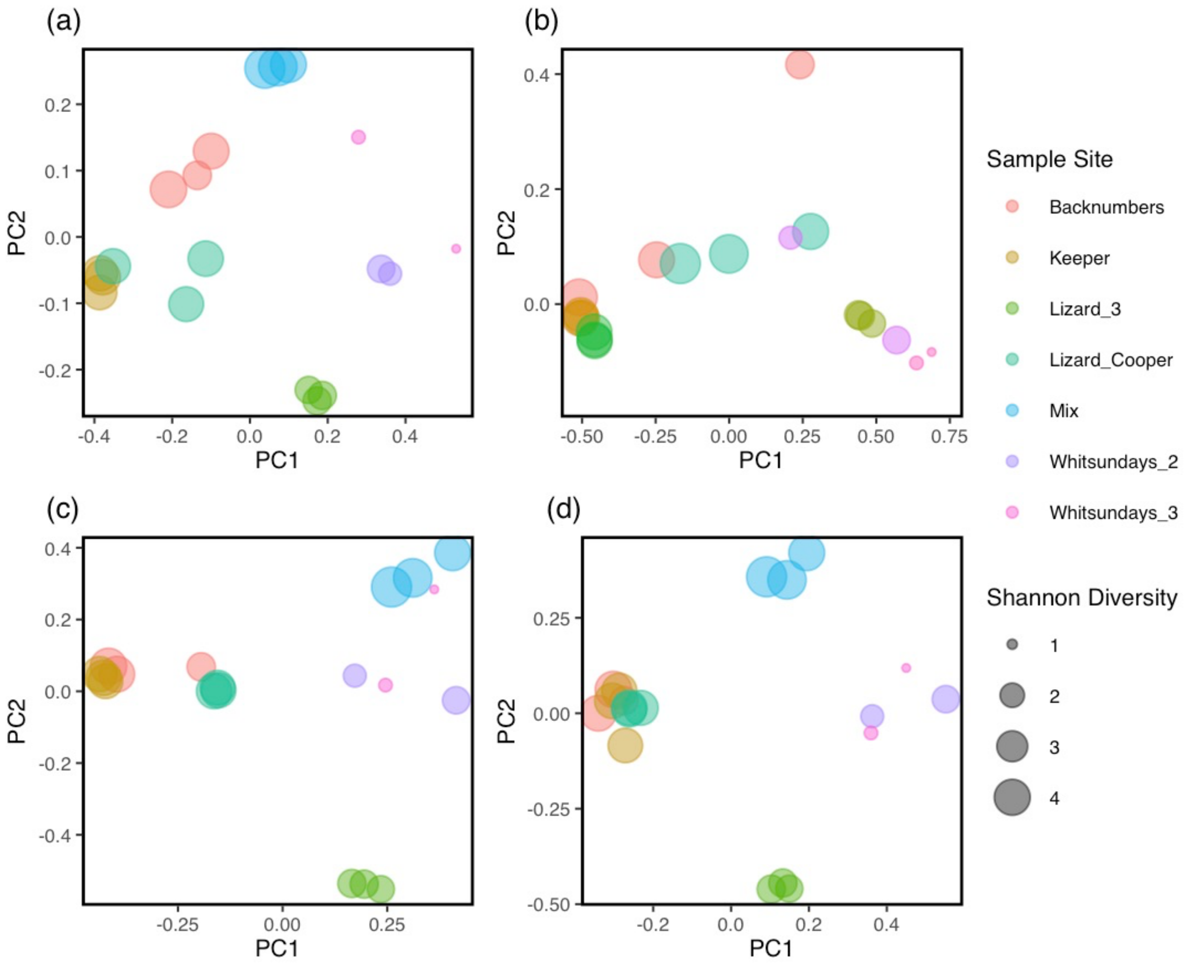


Figure 2.10 ITS2_acro PcoA plots with Shannon diversity index. Plots showing sample distribution based on (a) Unweighted Unifrac distance, (b) weighted Unifrac distance, (c) Bray-Curtis dissimilarity metric, (d) Jaccard dissimilarity. Colors represent reef sites and the known mix (blue). Dot size is proportional to Shannon Diversity Index, ranging from small dots representing lower diversity to larger dots indicating higher diversity.

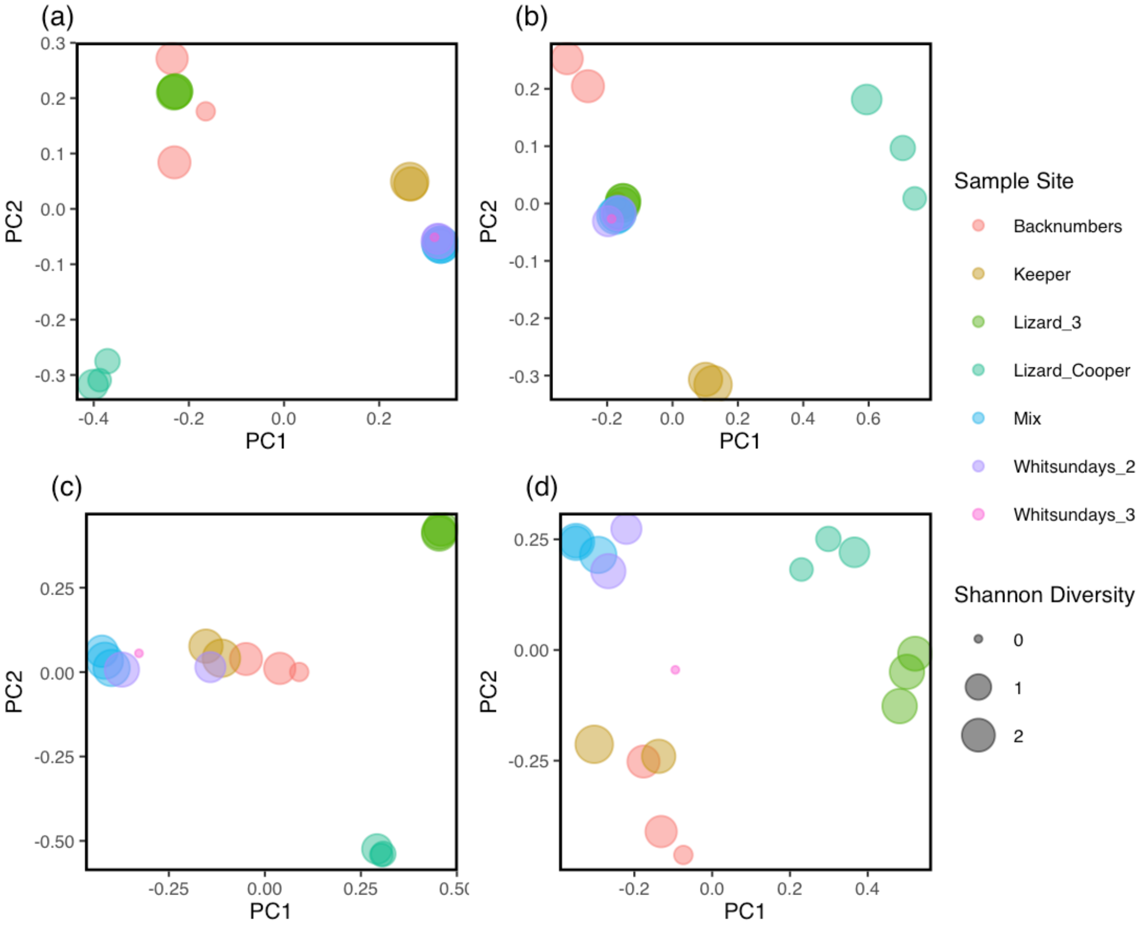


Figure 2.11 ITS2 PcoA plots with Shannon diversity index. Plots showing sample distribution based on (a) Unweighted Unifrac distance, (b) weighted Unifrac distance, (c) Bray-Curtis dissimilarity metric, (d) Jaccard dissimilarity. Colors represent reef sites and the known mix (blue). Dot size is proportional to the Shannon Diversity Index, ranging from small dots representing lower diversity to larger dots indicating higher diversity.

2.3.4) Assay Detection

The assay results show a variety of identified taxonomic groups, with some taxa not being resolved beyond the family and sub-order level. There was considerable cross-amplification of taxonomic groups across the two assays, and each assay identified unique families and genera (Figure 2.12, Figure 2.13). CoralITS2_acro identified a total of five families and six genera, while CoralITS2 identified seven families and 13 genera. While CoralITS2 detected more unique taxonomic groups, of the 13 genera identified by the CoralITS2 assay, five were not corals but rather sea anemones and sponges. Additionally, *Acropora* were exclusively detected with the CoralITS2_acro assay and represented 54% of the ASVs identified in this study, highlighting the importance of this revised primer set in detecting this reef-dominant species. Lastly, 23% of the ASVs identified to the family level could not be resolved to the genus level (NA), indicating that improved assay resolution could lead to more precise identification (Figure 2.13).

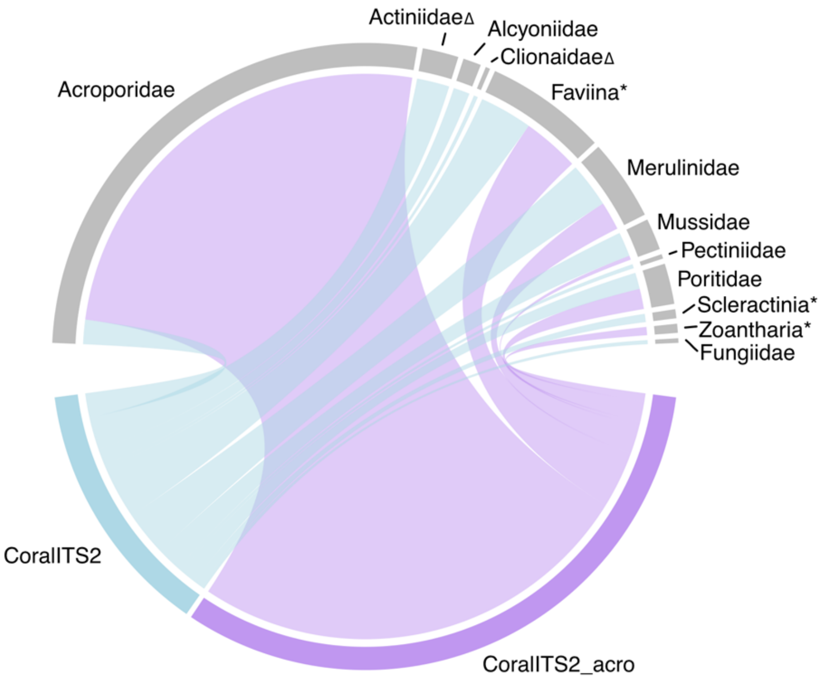


Figure 2.12 Taxonomic chord diagram of detections across CoralITS2_acro and CoralITS2 assays. Taxonomy is resolved to the family-level where possible, taxonomy that is not family is noted with an *. Non-coral detections noted with ∆. Size of endpoints reflects the number of unique ASVs attributed to each group.

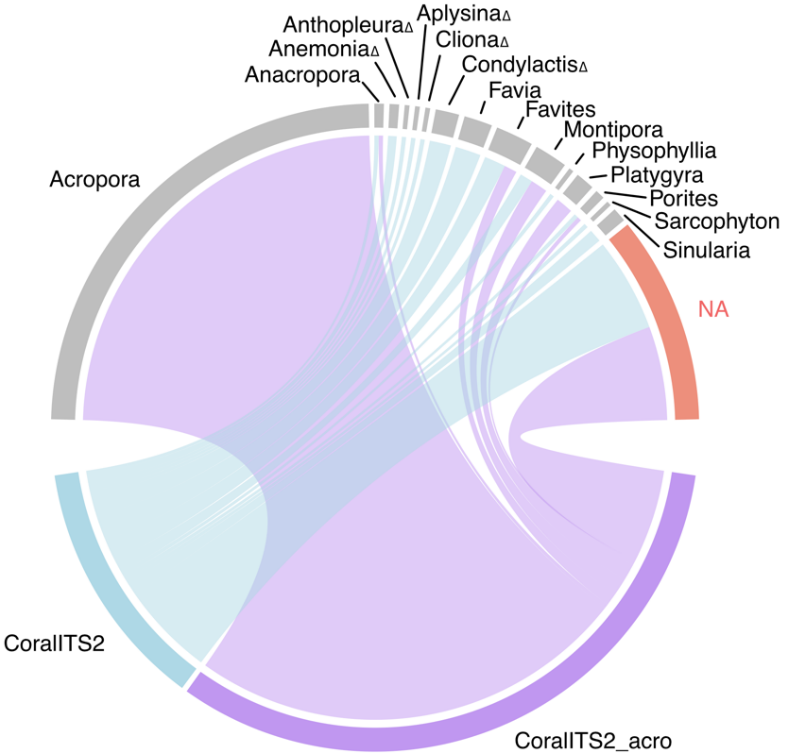


Figure 2.13 Taxonomic chord diagram illustrating detections across the CoralITS2_acro and CoralITS2 assays. Taxonomy is refined to the genus-level. NA represents ASVs that could not be resolved to the genus-level. Non-coral detections noted with ∆. Size of endpoints reflects the number of unique ASVs attributed to each genus.

2.4 Discussion

This study investigated the use of eDNA metabarcoding as a monitoring tool to improve wild spawning surveys and monitoring along the Great Barrier Reef. The survey recognized a total of 191 ASVs using the CoralITS2 and CoralITS2_acro assays. Creating a known mix consisting of equal ratios of 10 species of interest allowed for verification of each assay's detection capabilities, and relative abundance ratios of the metabarcoding product. While there was significant variation of relative abundance ratios among replicates, the Bayesian analysis using a beta-binomial model showed that the observed relative abundance values are consistent with the expected values, with no significant deviation detected. It was demonstrated that given the current capacity of coral genome databases, there is a trade-off between detailed resolution (genus-level) and the completeness of diversity representation (family-level). It was established that various PCoA can be used to investigate spawning slick similarities and differences incorporating phylogenetic trees, presence/absence, and count data. Furthermore, the observed overlap and differences in coral identification across assays indicate the value of integrating multiple techniques.

Incorrect taxa match, as seen in the observed known mix for CoralITS2, can arise from several factors such as hybridization (Willis et al., 2006) and database limitations (Dugal et al., 2022). Corals are known for their complex reproductive behaviors, including the formation of hybrids, or crossbreeding between different species. This can result in the sharing of genetic material across species, making it challenging to distinguish between corals based solely on genetic sequences, such as the ITS2 region. Hybrids may exhibit mixed genetic characteristics from their parent species, leading to ambiguous or incorrect taxa matches in sequence-based identification. Consequently, hybrids may be misclassified or fail to match precisely with reference sequences, leading to errors in identifying correct taxa. Additionally, taxonomic assignment accuracy and quality based on eDNA can only be as strong as the databases used for sequence matching. To address this, internal spiked datasets with individually extracted known samples can be created to enhance detection depth (Dugal et al., 2022). Addressing these gaps in taxonomic resolution could reduce the proportion of unresolved categories, and improve the accuracy of classification, thereby advancing the understanding of spawning slick biodiversity.

Factors leading to differences in relative abundance ratios between samples could be due to technical variations in the PCR process can significantly impact the measurement of relative abundance in DNA samples. Evidence suggests that variability in PCR outputs across different studies can be reduced by using multiple technical replicates, ideally between three and six, for each sample (Ficetola et al., 2015; Fonseca, 2018). This approach addresses inconsistencies and improves the reliability of the data. Averaging results from these replicates leads to more estimates of relative abundance and minimizes the impact of technical artifacts. Thus, we suggest future known mix investigations to include at least three, but optimally six technical extraction replicates per assay.

Several factors contribute to the mismatch between the exact expected known sample relative abundance and the observed outputs after PCR amplification, such as the variation in ITS2 sequence length and DNA not being amplified. ITS2 sequence length varies among coral taxa (Alexander et al., 2020). Shorter ITS2 sequences tend to be amplified more efficiently, and this may appear disproportionately abundant compared to longer sequences, due to the amplification bias inherent in PCR (Fonseca, 2018). Additionally, not all species present in the known samples may be adequately detected or amplified during PCR, leading to incomplete or skewed results. Aside from the single *Platygyra* added to our known mix, it is impossible to be certain all *Acropora* and others are being identified. To address this, it is crucial to test primers on pure culture samples to establish a comprehensive known library. This will ensure primers detect the desired populations accurately, thereby improving the accuracy of relative abundance assessments in mixed samples.

Displaying relative abundance to the family level ensures that all observations are captured, providing a comprehensive view of the community. However, analyzing the data at the genus level offers more detailed insights into community composition by identifying individual genera. This finer resolution, while beneficial for understanding specific genera, results in some loss of diversity metrics. At the genus level, taxa that were grouped at the family level may no longer be identifiable, leading to a reduction in the overall explained diversity. To improve genus-level identification while preserving overall explained diversity, enhancements to gene reference databases through increased genotyping efforts are necessary.

As the samples represent technical replicates from each reef location, high similarity within each reef location and among reefs with similar slick compositions was anticipated in the PcoA results. This expectation was confirmed as samples from the same reef location generally clustered closely together. When incorporating abundance data into the PcoA matrix calculations, the divergence between reef sites increased, suggesting that abundance information enhances the detection of distinct differences and temporal changes between reef sites of this data set. Overall, these findings demonstrate that PcoA, especially when combined with abundance data, is a valuable tool for monitoring spawning slick compositions.

While Principal Coordinates Analysis (PcoA) provides valuable insights into the clustering of reef samples and reveals patterns in community structure, it has limitations in elucidating the underlying drivers of these differences due to the constraints of our dataset. Specifically, the constraint of having only single samples collected at each reef results in analyses that are effectively technical replicates. To enhance the robustness of the analysis, it is suggested to collect samples from the same regions over multiple time points and simultaneously gather comprehensive environmental data such as wind fields, bleaching events, water temperature, and other relevant conditions during spawning (Keith et al., 2016; Lin & Nozawa, 2023; Nozawa, 2012; Van Woesik, 2010). Such data would enable a deeper understanding of how environmental factors influence community dynamics. Additionally, PcoA can be effectively utilized to monitor the impact of transplants and other restoration activities by assessing changes in the genetic makeup of reef populations over time and their effects on the spawning gene pool. It can also help characterize reproductive changes following bleaching events, where surviving corals are expected to experience reduced fecundity (Ward et al., 2002), as indicated by shifts in the relative abundance within spawning slicks. Furthermore, PcoA can elucidate shifts in spawning patterns, providing insights into how environmental factors influence the timing and distribution of spawning activities.

Leveraging eDNA methods for spawning monitoring offers several significant advantages. First, it enhances detection capabilities by allowing the simultaneous identification of multiple taxa, including those that are difficult to observe directly. This method can also confirm whether a spawning slick is composed of corals rather than other marine mass synchronized spawners that follow similar patterns, such as anemones. Additionally, eDNA is non-invasive and cost-effective. It reduces the need for extensive fieldwork and specialized training for accurate direct observations, thereby lowering monitoring costs and facilitating more frequent and widespread surveys. This approach also improves our understanding of spawning dynamics by enabling the collection of data on spawning times, species diversity, and genetic information. Furthermore, eDNA provides an opportunity to monitor changes in spawning patterns in response to environmental stressors and serves as a valuable tool for assessing the effectiveness of restoration activities, offering deeper insights into the health and behavior of marine ecosystems.

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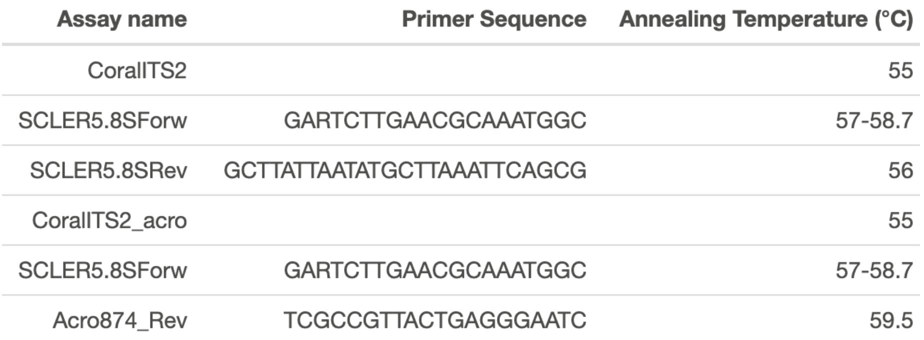
Supplemental Material

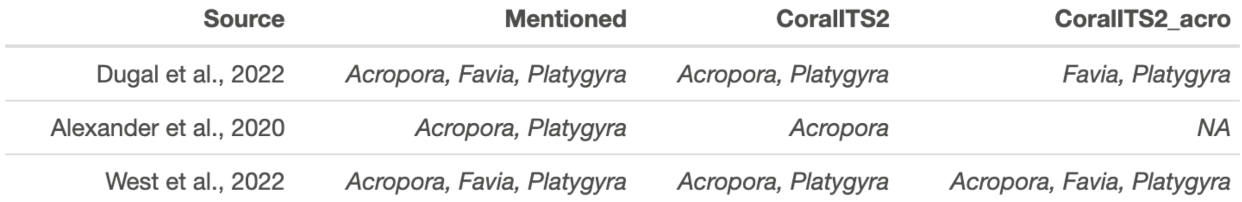
Supplementary Tables

Table S1. Primer pairs chosen for the ITS2 assays used in coral eDNA metabarcoding. The annealing temperature profiles were sourced from Alexander et al. (2020). CoralITS2 and CoralITS2_acro sequences were obtained from Brian, Davy & Wilkinson (2019), and Alexander et al. (2020) respectively.



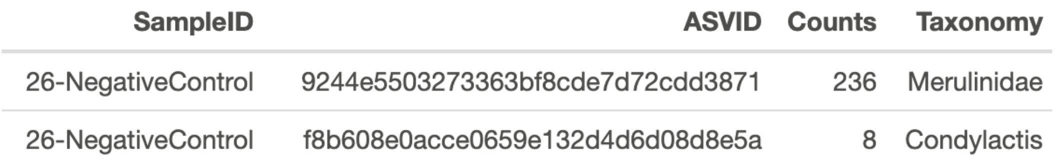
Assay name	Primer Sequence	Annealing Temperature (°C)
CoralITS2		55
SCLER5.8SForw	GARTCTTGAACGCAAATGGC	57-58.7
SCLER5.8SRev	GCTTATTAATATGCTTAAATTCAGCG	56
CoralITS2_acro		55
SCLER5.8SForw	GARTCTTGAACGCAAATGGC	57-58.7
Acro874_Rev	TCGCCGTTACTGAGGGAATC	59.5

Table S2. Summary of previous research that mentions species within our known mix, and whether that species was detected with CoralITS2 or CoralITS2_acro



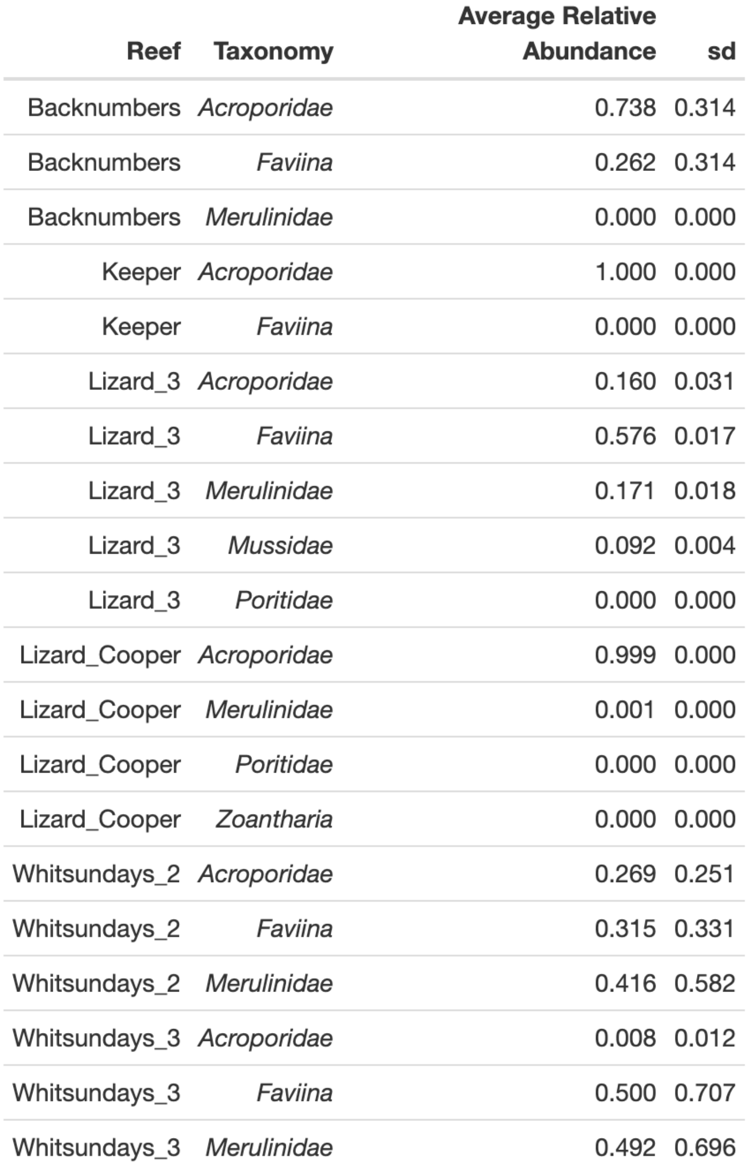
Source	Mentioned	CoralITS2	CoralITS2_acro
Dugal et al., 2022	*Acropora, Favia, Platygyra*	*Acropora, Platygyra*	*Favia, Platygyra*
Alexander et al., 2020	*Acropora, Platygyra*	*Acropora*	*NA*
West et al., 2022	*Acropora, Favia, Platygyra*	*Acropora, Platygyra*	*Acropora, Favia, Platygyra*

Table S3. Negative control results for the CoralITS2 assay. ASV identifiers (ASVID) detected in negative control were not detected in any other sample



SampleID	ASVID	Counts	Taxonomy
26-NegativeControl	9244e5503273363bf8cde7d72cdd3871	236	Merulinidae
26-NegativeControl	f8b608e0acce0659e132d4d6d08d8e5a	8	Condylactis

Table S4. Average relative abundance and standard deviation of each detected family taxonomy per wild sample reef site for the CoralITS2_acro assay

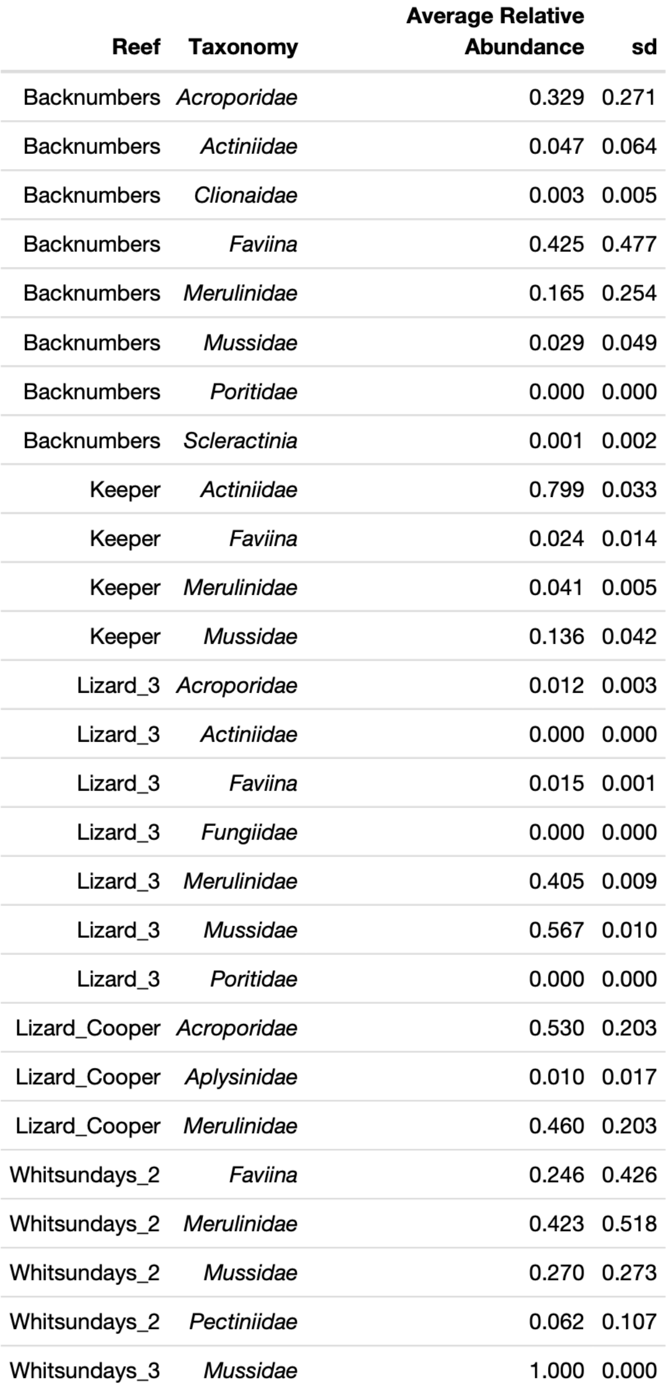


Reef	Taxonomy	Average Relative Abundance	sd
Backnumbers	*Acroporidae*	0.738	0.314
Backnumbers	*Faviina*	0.262	0.314
Backnumbers	*Merulinidae*	0.000	0.000
Keeper	*Acroporidae*	1.000	0.000
Keeper	*Faviina*	0.000	0.000
Lizard_3	*Acroporidae*	0.160	0.031
Lizard_3	*Faviina*	0.576	0.017
Lizard_3	*Merulinidae*	0.171	0.018
Lizard_3	*Mussidae*	0.092	0.004
Lizard_3	*Poritidae*	0.000	0.000
Lizard_Cooper	*Acroporidae*	0.999	0.000
Lizard_Cooper	*Merulinidae*	0.001	0.000
Lizard_Cooper	*Poritidae*	0.000	0.000
Lizard_Cooper	*Zoantharia*	0.000	0.000
Whitsundays_2	*Acroporidae*	0.269	0.251
Whitsundays_2	*Faviina*	0.315	0.331
Whitsundays_2	*Merulinidae*	0.416	0.582
Whitsundays_3	*Acroporidae*	0.008	0.012
Whitsundays_3	*Faviina*	0.500	0.707
Whitsundays_3	*Merulinidae*	0.492	0.696

Table S5. Average relative abundance and standard deviation (SD) of each detected genus taxonomy per wild sample reef site for the CoralITS2_acro assay with NA to represent taxonomy not resolvable to genus level

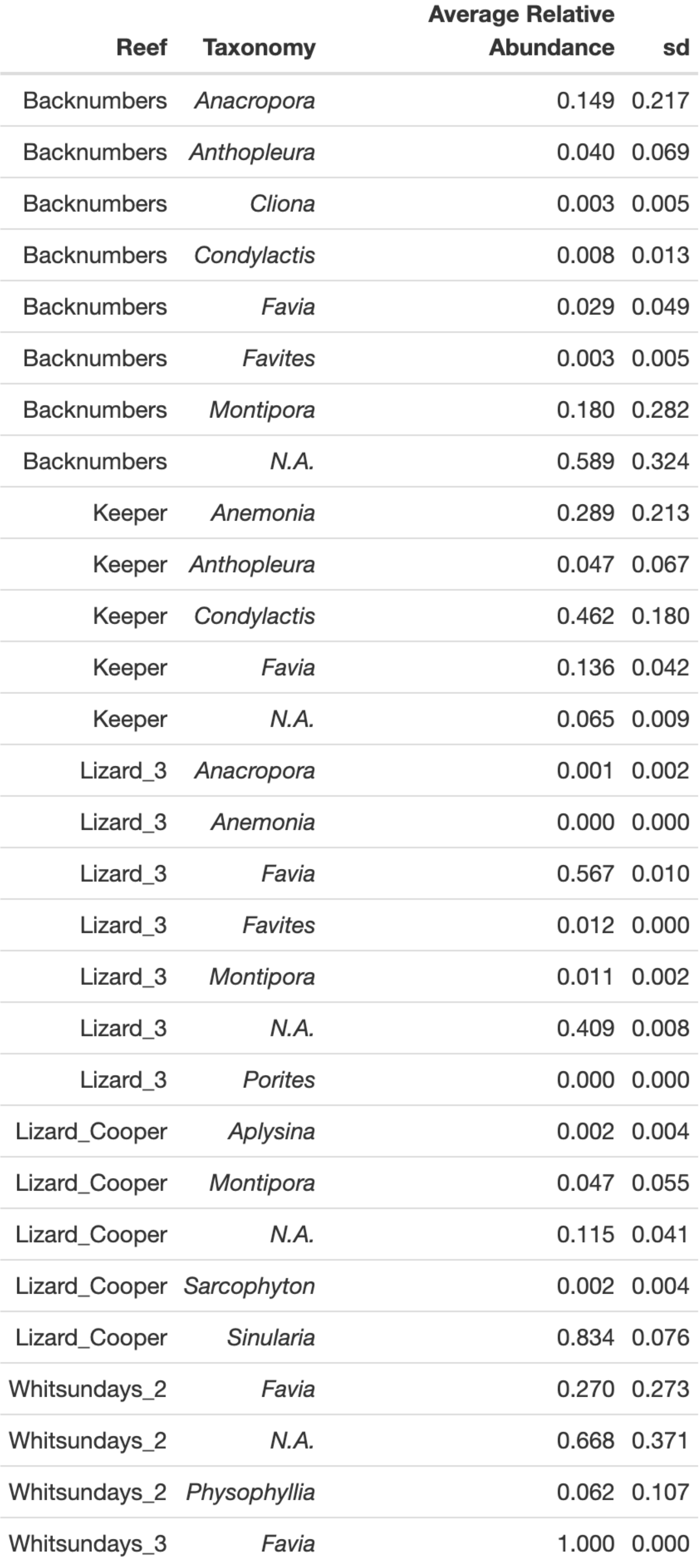
Reef	Taxonomy	Average Relative Abundance	sd
Backnumbers	*Acropora*	0.734	0.316
Backnumbers	*Anacroporapora*	0.001	0.001
Backnumbers	*Montipora*	0.002	0.003
Backnumbers	*N.A.*	0.262	0.314
Keeper	*Acropora*	1.000	0.000
Keeper	*N.A.*	0.000	0.000
Lizard_3	*Acropora*	0.152	0.031
Lizard_3	*Favites*	0.009	0.002
Lizard_3	*N.A.*	0.817	0.028
Lizard_3	*Platygyra*	0.023	0.002
Lizard_3	*Porites*	0.000	0.000
Lizard_Cooper	*Acropora*	0.999	0.000
Lizard_Cooper	*Montipora*	0.001	0.000
Lizard_Cooper	*N.A.*	0.000	0.000
Lizard_Cooper	*Platygyra*	0.001	0.000
Whitsundays_2	*Acropora*	0.269	0.251
Whitsundays_2	*N.A.*	0.540	0.020
Whitsundays_2	*Platygyra*	0.192	0.271
Whitsundays_3	*Acropora*	0.008	0.012
Whitsundays_3	*N.A.*	0.500	0.707
Whitsundays_3	*Platygyra*	0.492	0.696

Table S6. Average relative abundance and standard deviation of each detected family taxonomy per wild sample reef site for the CoralITS2 assay



Reef	Taxonomy	Average Relative Abundance	sd
Backnumbers	*Acroporidae*	0.329	0.271
Backnumbers	*Actiniidae*	0.047	0.064
Backnumbers	*Clionaidae*	0.003	0.005
Backnumbers	*Faviina*	0.425	0.477
Backnumbers	*Merulinidae*	0.165	0.254
Backnumbers	*Mussidae*	0.029	0.049
Backnumbers	*Poritidae*	0.000	0.000
Backnumbers	*Scleractinia*	0.001	0.002
Keeper	*Actiniidae*	0.799	0.033
Keeper	*Faviina*	0.024	0.014
Keeper	*Merulinidae*	0.041	0.005
Keeper	*Mussidae*	0.136	0.042
Lizard_3	*Acroporidae*	0.012	0.003
Lizard_3	*Actiniidae*	0.000	0.000
Lizard_3	*Faviina*	0.015	0.001
Lizard_3	*Fungiidae*	0.000	0.000
Lizard_3	*Merulinidae*	0.405	0.009
Lizard_3	*Mussidae*	0.567	0.010
Lizard_3	*Poritidae*	0.000	0.000
Lizard_Cooper	*Acroporidae*	0.530	0.203
Lizard_Cooper	*Aplysinidae*	0.010	0.017
Lizard_Cooper	*Merulinidae*	0.460	0.203
Whitsundays_2	*Faviina*	0.246	0.426
Whitsundays_2	*Merulinidae*	0.423	0.518
Whitsundays_2	*Mussidae*	0.270	0.273
Whitsundays_2	*Pectiniidae*	0.062	0.107
Whitsundays_3	*Mussidae*	1.000	0.000

Table S7. Average relative abundance and standard deviation (SD) of each detected genus per wild sample reef site for the CoralITS2 assay with NA to represent taxonomy not resolvable to genus level



Reef	Taxonomy	Average Relative Abundance	sd
Backnumbers	*Anacropora*	0.149	0.217
Backnumbers	*Anthopleura*	0.040	0.069
Backnumbers	*Cliona*	0.003	0.005
Backnumbers	*Condylactis*	0.008	0.013
Backnumbers	*Favia*	0.029	0.049
Backnumbers	*Favites*	0.003	0.005
Backnumbers	*Montipora*	0.180	0.282
Backnumbers	*N.A.*	0.589	0.324
Keeper	*Anemonia*	0.289	0.213
Keeper	*Anthopleura*	0.047	0.067
Keeper	*Condylactis*	0.462	0.180
Keeper	*Favia*	0.136	0.042
Keeper	*N.A.*	0.065	0.009
Lizard_3	*Anacropora*	0.001	0.002
Lizard_3	*Anemonia*	0.000	0.000
Lizard_3	*Favia*	0.567	0.010
Lizard_3	*Favites*	0.012	0.000
Lizard_3	*Montipora*	0.011	0.002
Lizard_3	*N.A.*	0.409	0.008
Lizard_3	*Porites*	0.000	0.000
Lizard_Cooper	*Aplysina*	0.002	0.004
Lizard_Cooper	*Montipora*	0.047	0.055
Lizard_Cooper	*N.A.*	0.115	0.041
Lizard_Cooper	*Sarcophyton*	0.002	0.004
Lizard_Cooper	*Sinularia*	0.834	0.076
Whitsundays_2	*Favia*	0.270	0.273
Whitsundays_2	*N.A.*	0.668	0.371
Whitsundays_2	*Physophyllia*	0.062	0.107
Whitsundays_3	*Favia*	1.000	0.000

Supplementary Figures

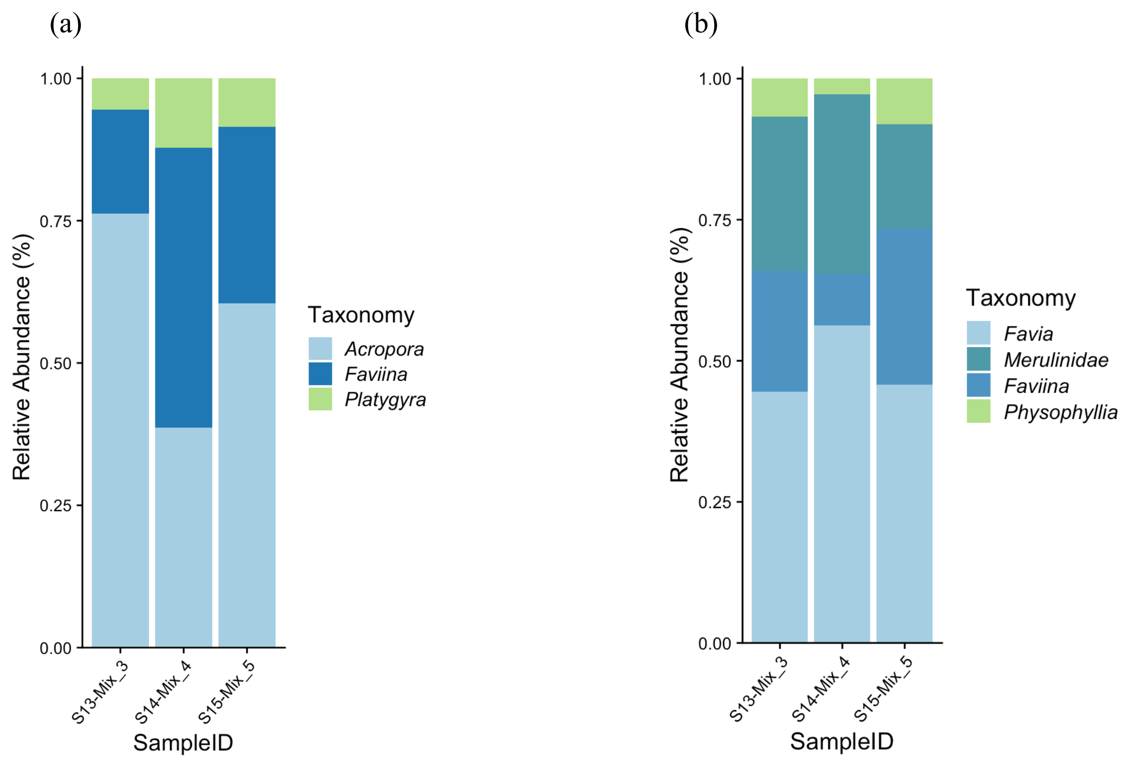


Figure S1. Bar plot showing the relative abundance of taxa for each known mixes sample of the (a) CoralITS2_acro and (b) Coral ITS2 assay.

Supplementary Bayesian Model Figures: Ensure model is a good fit

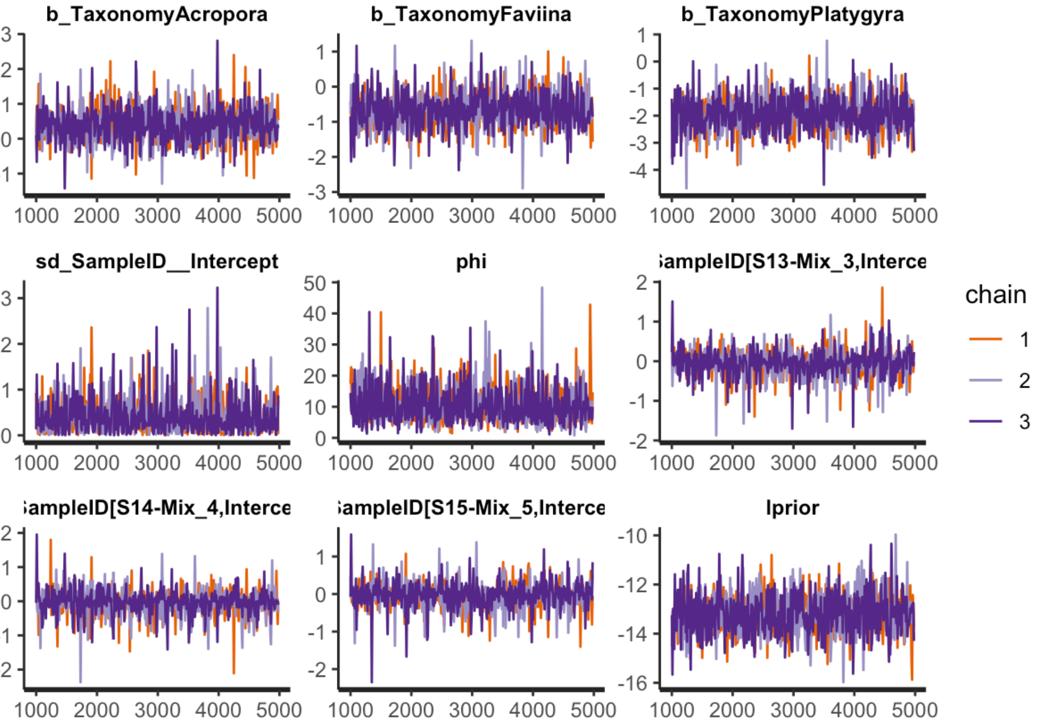


Figure S2. Traceplots of individual draws in sequence. Traces that resemble noise suggest all likelihood features are likely to have been traversed. Obvious steps or blocks of noise are likely to represent distinct features not yet traversed, necessitating additional iterations. Each chain should be indistinguishable from the others, confirmed by these plots.

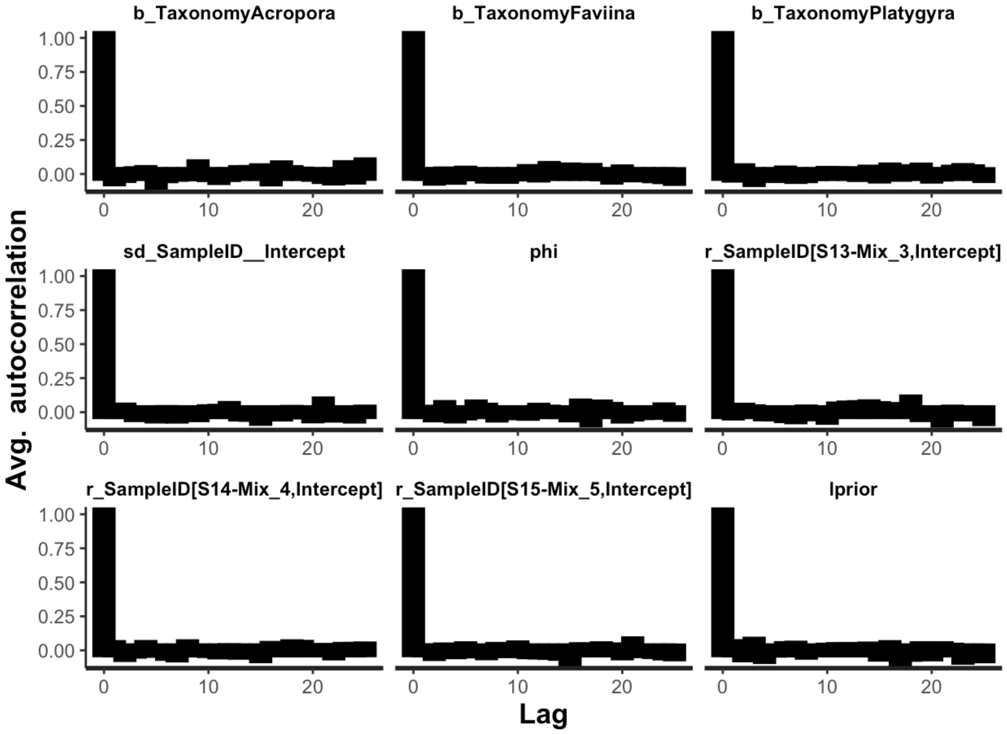


Figure S3. The autocorrelation function is expected to spike at 0 with a lag at 1, as shown here. If lag at 1 is not observed (i.e. bars slowly slope to 0), this would indicate autocorrelation between points, suggesting increased thinning. High levels of correlation (after a lag of 0) suggest a lack of independence between the draws therefore, summaries such as median would be biased estimates.

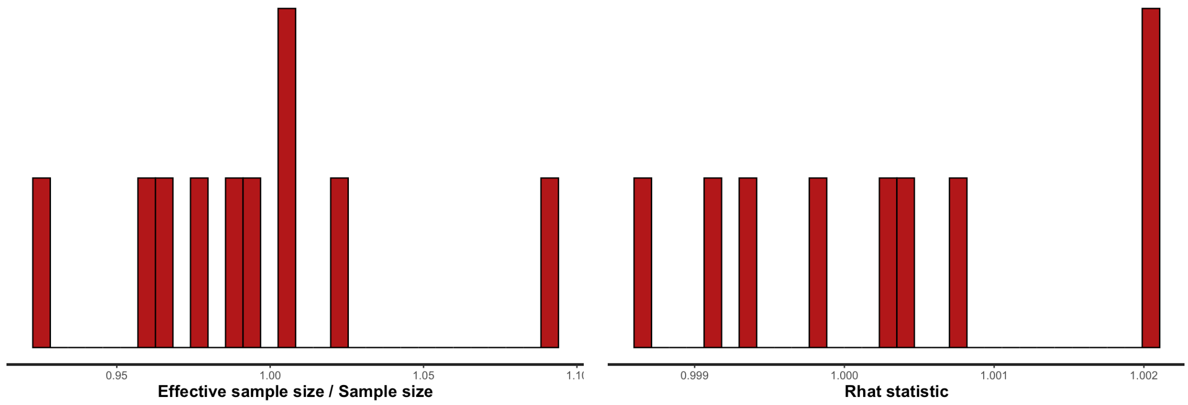


Figure S4. Effective sample size and Rhat statistic. Effective sample size/ sample size (ESS) values > 0.5 ensures good sampling speed. If values <0.5 this is an indication that the sampler was inefficient, and priors should be narrowed. Rhat values should be less than 1.01 to ensure sampling was effective. If values > 1.01 priors should be adjusted. The more one chain deviates from others, the higher the Rhat value. Values less than 1.01 are considered evidence of convergence. Our model is well mixed and converged as shown in these plots.

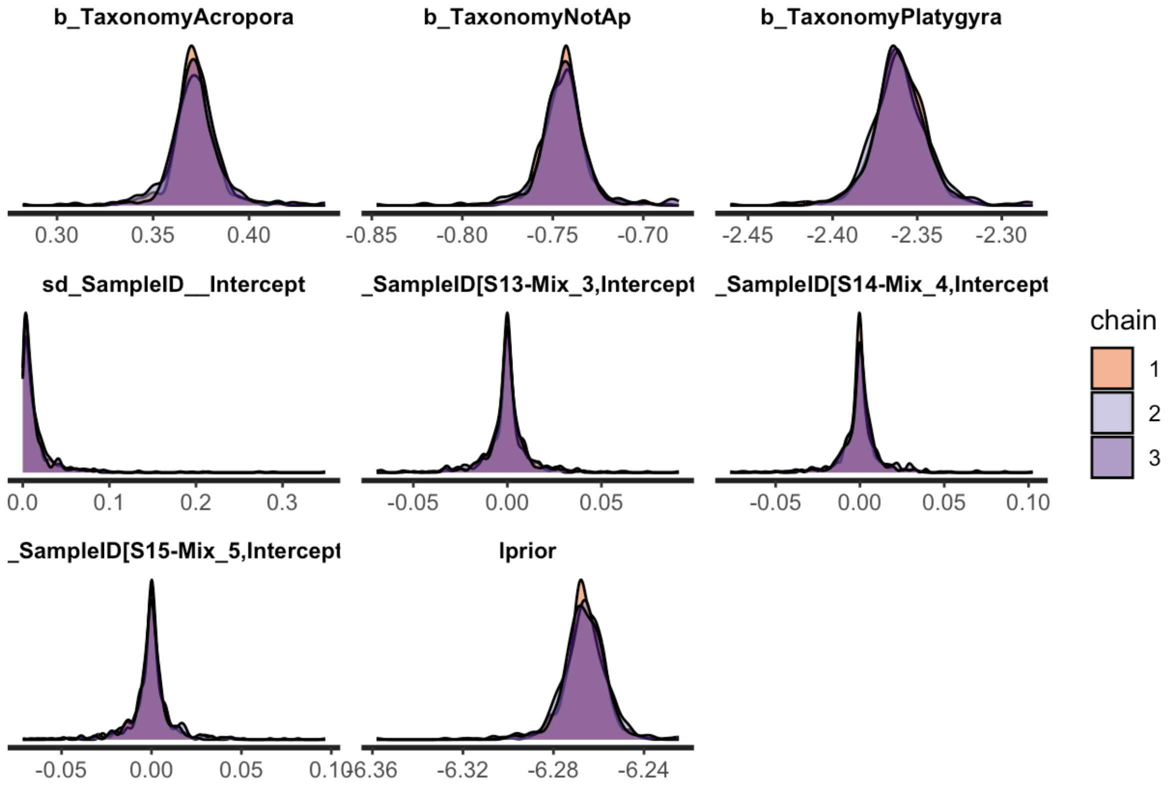


Figure S5. Density plots for each chain to ensure all three chains converged well, additional evidence to support of findings in Rhat statistic plots.

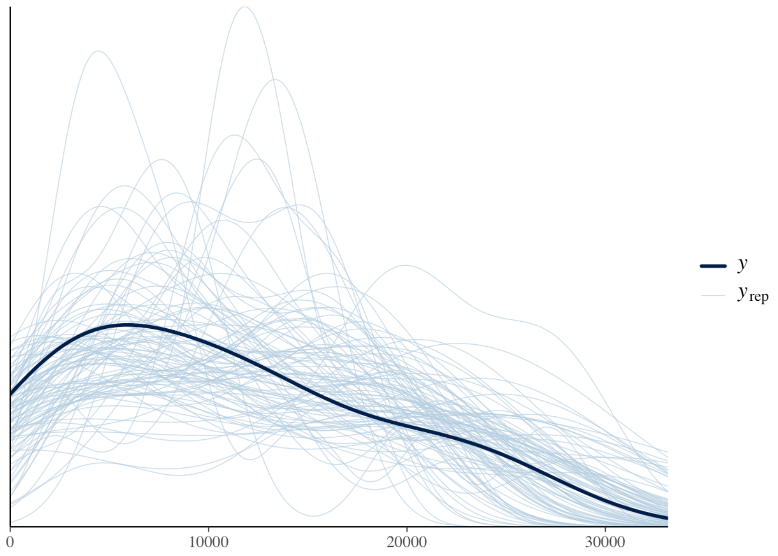


Figure S6. Posterior probability checks. y (dark line) is the distribution of relative abundance while y_{rep} (light blue lines) are the predicted distribution of relative abundance from the model (plotted 100 distributions).

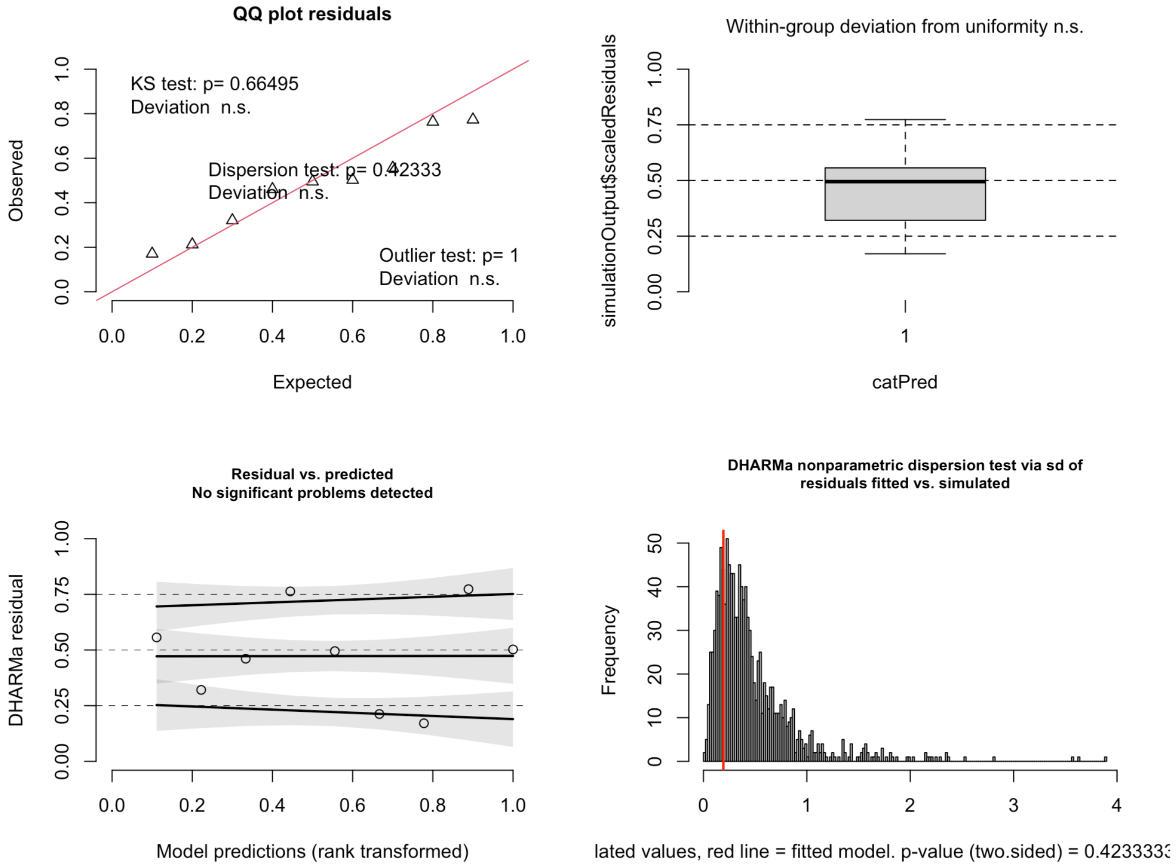


Figure S7. QQplot residuals (noted in Δ) should plot in a straight line as shown. Ensures normality of model curve. Residual plot, looking for a pattern, three horizontal lines mean data is expected