Coral Reefs
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REPORT

Comparing anesthetic stations and environmental DNA sampling to determine community composition of cryptobenthic coral reef fishes of Vava'u, Kingdom of Tonga

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Abstract Cryptobenthic reef fishes (CRF) are an important component of coral reef communities that are often overlooked, as their cryptic nature and very small size make them difficult to assess using visual methods. The prevailing method for quantifying communities of CRF is to use enclosures and anesthetics to collect fishes from within the reef. Environmental DNA (eDNA) sampling of water around reefs may be an alternative or supplemental way to quantify CRF communities. Here, we compare the ability to characterize CRF communities using eDNA sequences from water samples collected from within the interstices of the reef, with corresponding anesthetic stations from the same microhabitat. A total of 676 CRF from 33 species were collected from anesthetic stations, whereas only four species of CRF were detected from the eDNA samples taken at the same locations. Main factors contributing to these results may have been: (1) low standing biomass of CRF, thus low abundance of detectable DNA in the eDNA water samples; (2) large number of non-targeted metazoan reads that "drowned" the detections of specific CRF sequences; and (3) lack of sequences available in public databases that represent the high level of endemism present in CRF and a need for more complete taxonomic inventory.

Keywords Biodiversity · Metabarcoding · COI

Introduction

Coral reef ecosystems are biodiversity hotspots that house an estimated one quarter to one-third of all marine species (Plaisance et al. 2011). Despite coral reef fishes being a large proportion of this tremendous biodiversity (~6,000 species; Brandl et al. 2018; Eschemeyer et al. 2010), most reef biodiversity surveys overlook nearly half of this community by ignoring small and cryptic species that are important components of reef trophodynamics (Bierwagen et al. 2018; Bohmann et al. 2014; Brandl et al. 2018, 2019). The need to obtain a more comprehensive census of life in coral reef ecosystems has become urgent due to the imperiled status of the shallow reef building corals (McClenachan 2015) that house most of these 'less visible' reef fauna (Plaisance et al. 2009).

The smallest vertebrates on coral reefs are cryptobenthic reef fishes (CRF). Species of CRF belong to at least 17 different families of fishes, are typically less than 5 cm long, and live very close to the coral substrate, often hiding within the crevices of the reef (Depczynski and Bellwood 2004; Brandl et al. 2018). Their minute sizes allow them to exploit nearly all available microhabitats found in coral reef ecosystems. CRF can use these microhabitats in very specific ways, which may help explain the high species richness observed within this group (Brandl et al. 2019; Greenfield 2017, 2021; Winterbottom et al. 2014). CRF represent about 40% of the fish species present in coral reef ecosystems, and about 50%

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of fish abundance (Depczynski and Bellwood 2004, 2005, Brandl et al. 2018). Because of their high turnover rates (e.g., the goby *Eviota sigillata* has the shortest known vertebrate lifespan at only 59 d; Depczynski and Bellwood 2005), they transfer substantial energy as a constantly replenishing source of prey for larger reef inhabitants (Brandl et al. 2019). Consequently, there is a clear need to include CRF when quantifying community composition on reefs.

The most effective method for the study of CRF is to collect specimens from the reef using chemicals and euthanizing them so that they can be properly identified. These collections are key for contributing to studies on systematics as well as those looking at community structure, trophic ecology, and other ecological questions (Ahmadia et al. 2018; Atta et al. 2019; Brandl, et al. 2020a, b a,b; Troyer et al. 2018). Collecting cryptobenthic fishes requires the use of ichthyocides or anesthetics, often in combination with mesh and or plastic enclosures (Ackerman and Bellwood 2000; Robertson and Smith-Vaniz 2008, 2010; Depczynski and Bellwood 2004). Different chemicals may have varying levels of effectiveness on different species. Some of the more secretive species that are deep within the reef crevices or those less sensitive to the chemical solutions may still go undetected (Ackerman 2002). Moreover, identification after capture often requires high-quality color photographs of fresh specimens, examination under a microscope, and a moderate to high degree of taxonomic training coupled with available taxonomic literature; even then, molecular analysis may be required for some groups.

Sampling environmental DNA (eDNA) is an emerging technique for surveying biodiversity (Thomsen and Willerslev 2015). Environmental DNA can be defined broadly as the pool of DNA isolated from the environment (Pawlowski et al. 2020). Despite the perceived versatility of eDNA sampling for biodiversity studies, there are issues that affect the success of this type of sampling. The persistence of eDNA in the marine environment varies depending on the surrounding conditions (Collins et al. 2018). Higher water temperatures are correlated with higher degradation rates of fish-originated eDNA (Tsuji et al. 2017). Shorter fragments of shed DNA may be recovered more readily in the hotter tropical environments, and thus, it is often more fruitful to use a combination of genetic markers and primers that could capture these shorter, but more abundant eDNA fragments (Collins et al. 2018). Several PCR primers have been designed to specifically recover fish DNA, including those targeting mitochondrial 12S or 16S (Miya et al. 2015; Valentini et al. 2016; DiBattista et al. 2017; see Zhang et al. 2020, for a review of fish primers). Despite the versatility and popularity of the 12S and 16S for fish eDNA studies, these markers usually do not contain sufficient variation to identify sequences to species level or differentiate among rapidly diversifying and closely related taxa, which is the case for many groups within the CRF (Tornabene et al. 2015, Mabuchi et al. 2014, and Lin and Hastings 2013). Moreover, there are fewer sequences of 12S and 16S in public databases when compared to loci like mitochondrial cytochrome c oxidase subunit I (COI), which is the most abundant locus in GenBank (Clark et al. 2016) and BOLD (Ratnasingham and Hebert 2007) due to widespread use in DNA barcoding studies (Hebert et al. 2003). The number of COI sequences for bony fishes (Class Actinopterygii) in GenBank is four to five times larger than what is available for 12S or 16S (as of August 2021). Because of its high mutation rate, it is recommended for taxonomic assignments at the species level, with the added advantage of allowing for discrimination among haplotypes within species populations (Andújar et al. 2018). These factors make it particularly useful for CRF eDNA experiments. A standard barcoding fragment from the larger whole COI gene (658 bp out of a total of ~ 1500 bp) targeted by "Folmer primers" (Folmer et al. 1994) has been specifically helpful in taxonomic studies of many CRF groups including gobies (Winterbottom et al. 2014), blennies (Lin and Hastings 2013), clingfishes (Conway et al. 2020), basslets (Baldwin et al. 2016), and cardinalfishes (Mabuchi et al. 2014).

There have been several recent studies using eDNA to survey reef fishes (Table 1). DiBattista et al. (2017) collected eDNA water samples from the surface above reefs in the Red Sea and used fish-specific mitochondrial 16S primers. They found that only about half of the species present in their combined NCBI, custom 16S genetic database, or observed using underwater surveys, were detected in their eDNA samples. Only one CRF was detected at the species level, which was recovered from NCBI. Nguyen et al. (2020) conducted visual fish surveys and compared the results with the fish detected in eDNA samples collected 10–20 cm above the substrate. They used primers targeting COI from all metazoans (Leray et al. 2013). Despite detecting > 200 metazoan taxa previously recorded for the local databases, only 0.7% of the reads were assigned to bony fishes, with only two CRF species detected in their samples. Most recent studies targeting fishes (Table 1) detected a range of 0–59 CRF at the species level, but only Bessey et al. (2020, 2021) detected two species of the genus *Eviota* (denoted as *E.* sp.1 and sp. 2, as species-level matches were not found), which is problematic as the genus *Eviota* is the most specious genus of CRF (123 species; Greenfield 2021) and is frequently the most abundant group of CRF (Ahmadia et al. 2018). There is a clear need to explore new approaches to tackle CRF detection deficiencies in eDNA studies.

Given the low standing biomass of CRF (Brandl et al. 2019) coupled with their behavioral propensity for remaining closely associated with the benthos, it may be easier to detect their DNA closer to the substrate. No eDNA studies published to date have focused on detection of CRF by

Table 1 Studies published since 2017 using environmental DNA samples (eDNA) to detect fishes in tropical environments

Study	Locus	Taxa targetted by primers	Total volume filtered (ml)	Collected from	Taxonomic assignment criteria (% similarity cutoffs)	Raw reads recovered	Genetic database	CRF matches: genus (species)	*Eviota* matches: genus (species)	Region
This study	COI	Metazoan	13,900	Benthos (on or within substrate)	> 95% species	1.6 M	MARES + inhouse	0(4)	0(4)	Tonga
Gelis et al. (2021)	COI	Metazoan	112,000	Midwater + substrate	Not reported	2.8 M	BOLD (customized)	1(1)	0(0)	Indonesia
Nguyen et al. (2020)	COI	Metazoan	134,000	Benthos (10–30 cm above)	Not reported	14.38 M	GB	0(13)	–	Caribbean Sea
DiBattista et al. (2021)	16S	Fish (and invertebrates)	90,000	Surface (30 cm)	> 95% species	~9.4 M	GB	0(6)	0(0)	Arabian Gulf, Sea of Oman, & Western Arabian Sea
Bessey et al. (2021)*	16S	Fish	9,000	Surface (1 m)	> 90% genus, > 97% species	8.32 M	GB	6(7)	0(2)	Western Australia
Bessey et al. (2020)	16S	Fish	20,700	Surface	Not reported	12.95 M	GB	0(10)	0(2)	Northwestern Australia
West et al. (2020)	16S	Fish (and invertebrates)	250,000	Surface	> 95% genus, > 99% species	Not reported	GB + inhouse	5(18)	0(0)	Eastern Indian Ocean
Stat et al. (2018)	16S	Fish	24,000	Surface	> 95% genus, > 99% species	740,847	Inhouse	1(0)	0(0)	Southwestern Australia
Stat et al. (2017)	16S	Fish	9,000	Surface	> 98% class Actinopterygii	Not reported	GB	6(1)	0(0)	Western Australia
DiBattista et al. (2017)	16S	Fish	8,000	Surface (20 cm)	> 95% genus, > 98% species	250,145	GB + inhouse	2(1)	1(0)	Red Sea
Marwayana et al. (2021)	12S	Fish	113,000	Midwater (1 m above substrate)	Not reported	6.6 M	CRUX + GB supplemented	9(62)	1(5)	Indonesia
Polanco-Fernandez et al. (2021)	12S	Fish	30,000	Surface	> 90% genus, 100% species	Not reported	GB	6(16)	–	Caribbean Sea
Juhel et al. (2020)	12S	Fish	18,000	Midwater (10–300 m)	> 90% genus, > 98% species	299.48 M	EMBL**	35(59)	0(0)	West Papua, Indonesia
Sigsgaard et al. (2020)	12S	Fish (and other vertebrates)	72,000	Surface, Midwater (30 m)	> 93% genus, > 99% species	Not reported	GB + inhouse	8(3)	0(0)	Arabian Gulf

*9,000 ml were collected by active filtration, other samples in study were collected by passive filtration. Only one genus was reported as being detected by passive filtration only, and was not included on the count

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collecting in situ underwater eDNA samples from within the interstices of the reef substrate where shed DNA fragments may be more concentrated (Turner et al. 2015; Gelis et al. 2021), nor have they collected the fishes from the same microhabitats from where the eDNA samples were taken. Here, we focused on collecting eDNA samples from within the interstices in coral heads and rubble substrates, followed by collection of CRF from within the same sampled microhabitats. This approach should theoretically make it easier to detect CRF in eDNA samples, since defining traits for this group include their mostly continuous physical contact with the substrate and their preference for hiding in interstices of the reef (Depczynski and Bellwood 2004). The objective of this study was to determine if in situ eDNA water samples would be a feasible method to survey CRF as an alternative or supplement to anesthetic stations. We specifically addressed how eDNA sampling compares to anesthetic stations when used as a tool to quantify CRF communities.

Methods

Sampling collection

All samples were collected during December 2018–January 2019 at Vava'u, the northernmost group of islands in the Tongan Archipelago (Fig. 1). See supplementary material for site description. At each site, we sampled three different microhabitat types: live coral bommies (LC), dead coral rock (DC), and coral rubble (CR) (see supplementary material for descriptions). The number of combined samples across sites for the live coral, coral rubble and dead coral microhabitats was eight, seven, and six, respectively (Table 2).

Environmental DNA and CRF samples were collected underwater using standard SCUBA gear. When a suitable microhabitat was selected, eDNA samples were either collected at the substrate surface (when interstices were absent), or within the interstices of the coral rubble or the coral heads, using 200 ml modified syringes (Figure S1), allowing for the rapid collection of eDNA samples, thus reducing the potential for including water from the surrounding environment (See supplementary material for detailed methods). Two to three replicates were taken from each station, with combined volumes ranging from 200–1200 ml per station. We restricted the amount of water that we sampled from each station because our objective was to capture the volume of water within the confines of the crevices of the microhabitats, or as close as possible to the substrate where the CRF are found. Water samples were filtered, and filter discs were preserved in Longmire's solution (Longmire et al. 1997). Immediately after collecting the eDNA samples, we collected CRF from that same microhabitat using enclosed anesthetic stations following protocols modified from Ahmadia et al. (2018), photographed specimens in the field, preserved them in 95% ethanol and identified them back in the laboratory (see Supplementary Material for details).

eDNA lab processing

DNA from each whole filter disc corresponding to individual sample replicates was extracted and kept as a separate sample for the subsequent amplification and sequencing processing. See supplementary material for details on DNA extraction. Extracted raw DNA from all the eDNA samples was sent to Jonah Ventures Lab (Boulder Colorado, USA) for sample amplification. A 313 bp section of the mitochondrial COI gene was selected and amplified via PCR using the forward UniCO1F and reverse UniCO1R primers (Leray et al. 2013). This smaller COI section was selected based on the following four premises: (i) it is located within the 658 bp 'Folmer' region, which is the mostly widely recognized universal marker for species identification (Herbert et al. 2003), making it the most well-represented marker in public molecular databases like GenBank (NCBI) and BOLD; (ii) when dealing with eDNA samples in warm tropical waters, a shorter segment of the COI would be more abundant than the complete 658 bp fragment used in DNA barcoding studies; (iii) COI (vs. 12S or 16S) has been used successfully to identify CRF species at the species level, and it has proven particularly useful for resolving taxonomic delineation of the genus *Eviota* and *Trimma* (dwarf gobies and pigmy gobies, respectively), two of the most abundant and diverse groups of CRF; (iv) the availability of an extensive inhouse *Eviota* COI sequence database spanning much of the Indo-Pacific region (Tornabene, et al. 2021, 2013a, b, 2015, 2016; Tornabene unpublished data). A 2-step PCR process was used to amplify target amplicons and identify unique samples, using standard laboratory procedures. Full details and concentrations can be found in Supplemental materials. The final library was run using a V2-500 cartridge on an Illumina MiSeq.

Bioinformatics and taxonomic assignments

The bioinformatic pipeline used for the cleaning and filtering of sequenced reads can be found in the GitHub repository—https://github.com/ramongallego/Nextera_Dada2. We chose the well-known sequence similarity method, BLAST (Altschul et al. 1997), which has been demonstrated to perform well as a taxonomic assignment tool (Hleap et al. 2021 – See supplementary material for details on the bioinformatic pipeline and the parameters used for taxonomic assignments).

To increase classification sensitivity for taxonomic assignments, a custom reference sequences database was prepared using MARES pipeline (Arranz et al. 2020). MARES includes all the COI sequences from GenBank

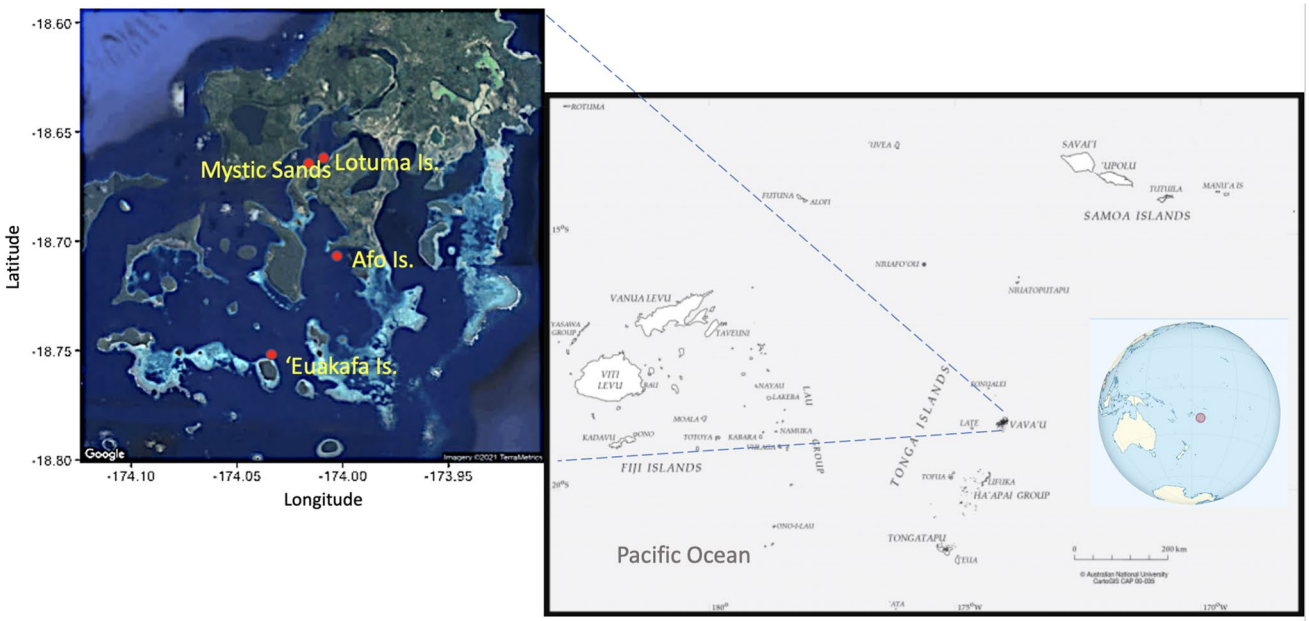

Fig. 1 Sampling sites in the Vava'u Island group (red dots in the inset map)

Table 2 Sampling effort and site coordinates for the eDNA and anesthetic stations

Site	Site coordinates		Cospatial eDNA and anesthetic stations			
	Latitude	Longitude	Live coral	Dead coral	Coral rubble	**Totals**
Lotuma Island	18°39′43.0"S	174°00′32.0"W	4	1	1	**6**
Mystic Sands	18°39′53.9"S	174°00′57.7"W	2	4	2	**8**
Afo Island	18°42′30.1"S	173°59′47.3"W	2	–	3	**5**
Euakafa Island	18°45′14.7"S	174°02′12.9"W	–	1	1	**2**
Total samples (ml of water)			**8 (5,500)**	**6 (2,800)**	**7 (5,400)**	**21 (13,900)**

Last row for "Total" information includes in parenthesis the combined volume of water (ml) filtered for eDNA analysis from each microhabitat

and BOLD for marine eukaryote taxa. Three important features of MARES are: (1) it restricts the number of sequences to just taxa from eukaryote families that are represented in the marine environment, making the querying process more efficient; (2) allows to add custom unpublished sequences; (3) a taxonomic normalization step synonymize information from BOLD, GenBank and custom sequences. Marine eukaryote sequences were retrieved from both GenBank and BOLD on July 17, 2021. For our study, and to supplement the reference database produced by MARES, we merged our "in-house" *Eviota* COI sequences database, which contains 460 sequences, with most (~75%) not yet uploaded to either GenBank or BOLD, each with a photographed voucher specimen. This *Eviota* database contains COI fragments (each ~658 bp) from an extensive and ongoing collecting effort from the entire Indo-Pacific region, with most specimens sampled from the Coral Triangle region, and lesser representation (2–20 specimens for each location) from Fiji, the Maldives, Moorea, Pohnpei, American Samoa, Solomon Islands, northeast Australia, and the Red Sea. This database also included 68 new COI sequences from the Vava'u Island group, Tonga, collected in 2018. Most of the sequenced specimens from Vava'u were part of the *Eviota* genus, yielding 16 species, 10 of them undescribed.

A final identification of each ASV was produced with a custom R script which used the LCA function from the R package taxonomizr to calculate the last common ancestor of all matching sequences in the database with a similarity of > 95% over at least 250 bp (See supplement for more detail). All statistical analyses and data visualization were done with R version 4.0.3.

Results

eDNA samples

The primers used in this study successfully aligned within the fish COI sequences available in the used reference database (Figure S2). A total volume of 13,900 ml were filtered from the 21 samples spread across three microhabitats (Table 2). The total number of raw sequences captured from all 21 eDNA samples after sequencing was 1,686,370. The read depth values for all the eukaryotes detected ranged between 9,186 and 91,613 per sample (combined replicates) with an average of 46,866 per sample (Table S1). After bioinformatic filtering using our combined MARES and *Eviota* database for taxonomic matching, a total of 313,184 marine eukaryote reads remained (Table 3). Among these reads, only 1,812 were assigned to CRF, belonging to four species from a single family, Gobiidae (Table 4). All four species belong to the genus *Eviota*, and three of the four species are undescribed: *Eviota* Tonga sp. 1 (1392 reads), *E.* Fiji sp. 1 (404 reads), *E. cf. punctulata* (8 reads), and *E.* Papua New Guinea sp. 1 (8 reads). Other non-CRF coral-reef fishes (19 species, 8 families) were detected for a total of 2,039 reads. A total of 1,105 of these reads belonged to *Naso brevirostris* and came from a single sample. A total of 1,964 reads from 19 ASVs could only be assigned to class Actinopterygii, and 4 reads from 1 ASV could only be assigned to class Chondrichthyes.

Anesthetic station samples

A total of 689 specimens belonging to 15 families (46 species) were collected from the 21 anesthetic station where eDNA samples were also taken (Table 4 & Figure S3). The number of specimens collected in each anesthetic station ranged from 2 to 99. Seven of the 17 core CRF families, as defined by Brandl et al. (2018), were represented by the 676 CRF specimens collected. The family Gobiidae made up the bulk of all fishes and CRF with 664 specimens. Only 10 specimens belonging to eight species distributed among six other CRF families were found (Table 4): Apogonidae (2), Blenniidae (2), Plesiopidae (1), Oxudercidae (1), Pseudochromidae (1), and Syngnathidae (1). Within the Gobiidae, the genus *Eviota* was about half of all CRF species, with 42% of all CRF belonging to one single species, *E. Tonga* sp. 1, which had 291 specimens collected from the combined three microhabitat types. This species along with *E. Tonga* sp. 2, and *Eviota cf. punctulata*, both also present in the eDNA samples, belong to the *Eviota sigillata* and to the *Eviota punctulata* species complexes, respectively, and are currently undergoing taxonomic revision. The same is the case for the *E.* Fiji sp. 1, belonging to the *E. fasciola* species complex, and *E.* Papua New Guinea sp. 1, belonging to the *E. melasma* species complex.

Anesthetic stations and eDNA comparisons

In total, 33 CRF species in 16 genera and 9 families were captured using eDNA and anesthetic stations. Eight other families of non-CRF were also recorded mainly from eDNA samples, with the family Pomacentridae representing 50% of all non-CRF species. The only CRF species that was found in both eDNA and anesthetic stations was *E. Tonga* sp. 1. It is made up 76% of all the CRF reads detected (1812) in eDNA samples, with more than half of them recovered from the coral rubble microhabitat. In the anesthetic stations, *E. Tonga* sp. 1 was also found in larger numbers in the coral rubble (46% of all specimens), as compared to the other two microhabitats combined. The other three species of CRF, *E. Fiji* sp. 1, *E. cf. punctulata, and E. Papua New Guinea* sp. 1, were not collected from anesthetic stations. The combined total species richness of CRF from coral rubble microhabitats was low (7) as compared to live coral (17), or dead coral microhabitats (19). In contrast, for eDNA samples detected more species from coral rubble microhabitats (11) than from live coral (3) and dead coral (7), as compared to the cospatial anesthetic station, with 10 species in coral rubble, 20 in live coral, 20 in dead coral (Fig. 2 and Figure S4).

There was no relationship between the number of fish reads detected in eDNA samples and the abundance of fishes collected from anesthetic station (Fig. 3). This was true for both CRF and non-CRF species. For eight out of the 21 stations, zero fish reads were detected in the eDNA samples,

Table 3 Summary of eDNA sequences recovered from entire study

	Number of reads	Percent (from initial raw reads)	Distinct ASVs	Unique taxa (fish species)
Total raw sequences	1,686,370	100	–	–
Marine eukaryotes*	313,184	18.6	1,211	347
Actinopterygii	3,851	0.2	39	22
Cryptobenthic Fishes	1,812	0.1	14	4

(*) Using MARES + Eviota database

Table 4 Fish species collected from cospatial anesthetic stations (AS) and eDNA samples

Non–CRF—family	Non–CRF—species	eDNA samples where species were detected	AS where species were collected	Reads in eDNA samples	Fishes in AS	Available COI seqs	
						Genus	Species
Apogonidae	*Cercamia eremia*	0	1	0	1	Y	Y
	Fowleria marmorata	0	1	0	1	Y	Y
Blenniidae	*Atrosalarias fuscus*	0	2	0	2	Y	Y
	Ecsenius bicolor	0	1	0	1	Y	Y
Gobiidae	*Amblygobius sphynx*	0	1	0	1	Y	Y
	Asterropteryx semipunctata	0	10	0	53	Y	Y
	Cabillus lacertops	0	1	0	1	Y	Y
	Cabillus cf. lacertops	0	2	0	2	Y	Y
	Eviota albolineata	0	1	0	1	Y	Y
	Eviota cf. punctulata	1	2	8	4	Y	Y
	Eviota cometa	0	2	0	5	Y	Y
	Eviota Fiji sp. 1	4	0	404	0	Y	Y
	Eviota herrei	0	2	0	4	Y	Y
	Eviota Papua New Guinea sp. 1	2	0	8	0	Y	Y
	Eviota prasites	0	4	0	41	Y	Y
	Eviota sebreei	0	4	0	6	Y	Y
	Eviota smaragdus	0	1	0	1	Y	Y
	Eviota Tonga sp.1	8	11	1,392	291	Y	Y
	Eviota Tonga sp. 2	0	3	0	4	Y	Y
	Eviota Tonga sp. 3	0	1	0	1	–	–
	Eviota sp. (juvenile)	0	3	0	3	–	–
	Eviota sparsa	0	9	0	31	Y	Y
	Eviota tigrina	0	12	0	129	Y	Y
	Fusigobius cf. aureus	0	3	0	3	Y	Y
	Fusigobius signipinnis	0	2	0	4	Y	N
	Gobiidae sp (juvenile)	0	10	0	33	–	–
	Grallenia sp.	0	1	0	1	N	N
	Koumansetta rainfordi	0	7	0	14	Y	Y
	Trimma benjamini	0	7	0	32	Y	Y
Oxudercidae	*Gnatholepis cauerensis*	0	1	0	1	Y	Y
Plesiopidae	*Plesiops Oxycephalus*	0	1	0	1	Y	N
Pseudochromidae	*Cypho purpurascens*	0	3	0	4	Y	Y
Syngnathidae	*Festucalex gibssi*	0	1	0	1	Y	N
	CRF totals	**17**	**13***	**1,812**	**677**		
Non-CRF—family	**Non-CRF—species**						
Acanthuridae	*Naso brevirostris*	1	0	1,105	0	Y	Y
Chaetodontidae	*Heniochus chrysostomus*	1	0	595	0	Y	Y
Labridae	*Halichoeres trimaculatus*	1	0	35	0	Y	Y
	Iniistius melanopus	1	0	30	0	Y	Y
	Labridae sp.	0	1	0	1	–	–
Pomacentridae	*Chromis viridis*	1	0	45	0	Y	Y
	Pomacentrus burroughi	0	3	0	3	Y	Y
	Pomacentrus callainus	1	0	9	0	Y	Y
	Pomacentrus lepidogenys	1	0	6	0	Y	Y
	Pomacentrus maafu	1	0	5	0	Y	Y
	Pomacentrus moluccensis	1	0	14	0	Y	Y

Table 4 (continued)

Non–CRF—family	Non–CRF—species	eDNA samples where species were detected	AS where species were collected	Reads in eDNA samples	Fishes in AS	Available COI seqs	
						Genus	Species
	Pomacentrus microspilus	1	0	4	0	Y	Y
	Pomacentrus nagasakiensis	1	0	16	0	Y	Y
	Pomacentrus sp. (juvenile)	0	3	0	7	–	–
Scombridae	*Rastrelliger kanagurta*	1	0	30	0	Y	Y
Serranidae	Serranidae sp. (juvenile)	0	1	0	1	–	–
Scorpaenidae	*Scorpaenodes hirsutus*	0	1	0	1	Y	Y
Synodontidae	*Saurida gracilis*	1	0	145	0	Y	Y
Unidentified	Unidentified	0	2	0	4	–	–
	Non-CRF totals	**13**	**11***	**2,039**	**NA**		

For the eDNA samples, only the species with > 95% identity from the bioinformatic processing were included. For the AS, all fish collected were included

*Some of the counts may be overlaps from same sample

while the number of fishes collected in corresponding anesthetic stations ranged from 10–62. The highest number of fish collected from a single anesthetic station was 99 (95 CRF), from a dead coral head, while 35 CRF reads were detected in the corresponding eDNA sample. In another anesthetic station (also a dead coral head microhabitat), a total of 9 fishes were collected (8 of them CRF).

Discussion

Analysis of eDNA from water collected from within the matrix of the reef was not an effective technique to survey the CRF community. Only 0.6% of all reads recovered from our eDNA samples were assigned to a total of 4 species of CRFs, compared to the 33 species found in the anesthetic stations. Except for two eDNA studies (Marwayana et al. 2021; Juhel et al. 2020) that documented ~ 60 CRF species from the Coral Triangle (Indonesia), most eDNA studies studying fishes report zero to 18 CRFs (Table 1). Our study is the first to focus on CRFs by collecting both eDNA samples and specimens from within reef microhabitats, whereas most other eDNA studies focus on reef fishes more broadly. Nevertheless, the many factors complicating the use of eDNA to detect CRF are shared across eDNA studies.

Two obvious factors that contribute to the detectability of target organisms in the environment are the size and abundance of the organism itself, and the rate at which the organisms are shedding DNA into the environment (Barnes and Turner 2016). Despite their very high abundances relative to the total coral reef fish community (50% of all individuals, Brandl et al. 2018), CRFs have body sizes ranging from less than 1 cm to 5 cm total length and only represent a very small slice (< 5%) of the total standing stock fish

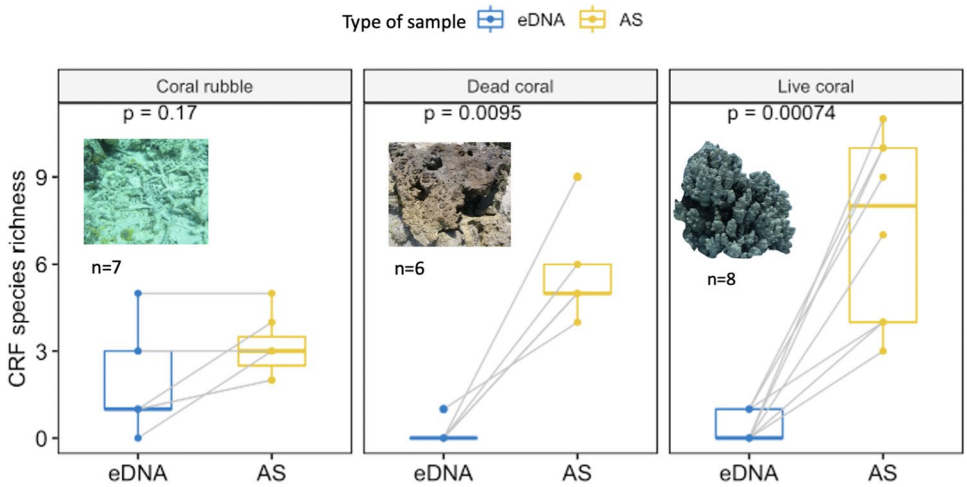

Fig. 2 Cryptobenthic reef fishes (CRF) species richness of eDNA and anesthetic stations (AS) samples for the three microhabitats sampled. Grey lines show CRF species richness for the eDNA and corresponding AS samples

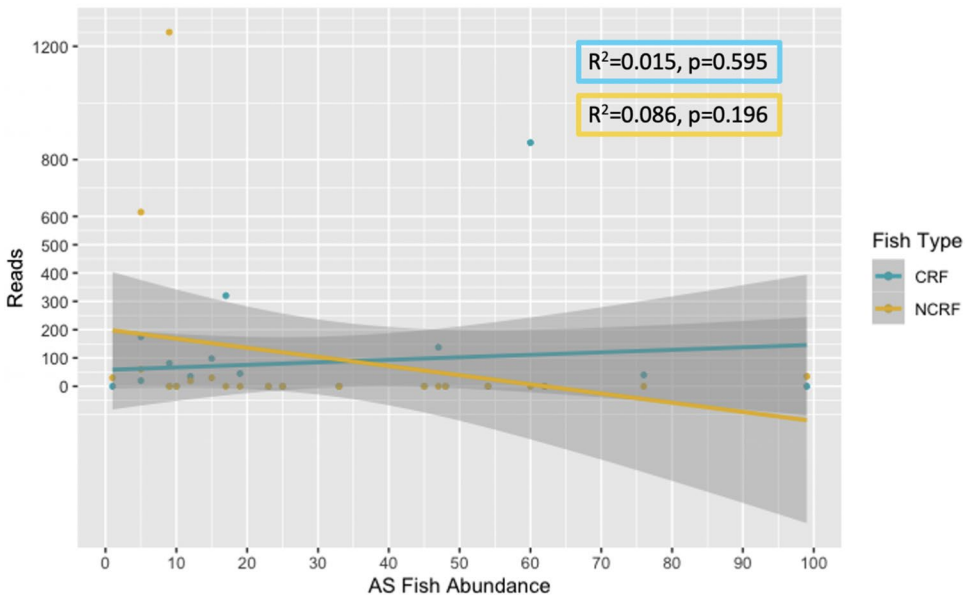

Fig. 3 Read abundance for cryptobenthic reef fishes (CRF) and non-CRF in relation to the number of fishes collected from cospatial anesthetic stations (AS)

biomass at any given moment on reefs (Brandl et al. 2019). Thus, shedding of DNA material from CRF must be minimal. This would include indirect contributions to the system from other coral dwellers that ingested CRF and dispersed their DNA in feces.

The number of reads in eDNA samples may not directly correlate with the abundance of organisms present in the sampled environment, due mostly to uneven amplification efficiency for different taxa during PCR process (Collins et al. 2019; Shelton et al. 2016; Kelly et al. 2017, 2019), and this holds true for our samples overall (Fig. 3). However, the most abundant species in our anesthetic stations (*E.* Tonga sp. 1291 specimens) also had the most reads in the eDNA samples (1392). On the other hand, no specimens of *E.* Fiji sp.1 were captured in anesthetic stations, while 404 reads from this species were detected in eDNA samples. A possible explanation for this mismatch could be the known primer bias amplification, which is common when using COI (Deagle et al. 2014), but it could also be attributed to the patchiness and random distribution of DNA particles in the environment (Barnes and Turner 2016). The low amounts of CRF DNA detected overall and the high variability in the number of reads present in our samples (0–860 reads from CRFs per sample) speaks to the patchiness of target DNA present in the environment, and the need to optimize lab processing of the samples so a more reliable and homogeneous picture of all the CRF present in the surveyed sites is accomplished.

It is likely that eDNA from CRF is indeed incredibly rare around reefs and therefore, requires copious amounts of water for reliable detection, especially in warm tropical waters (Marwayana et al. 2021). Marwayana et al. (2021) recommended that as much as 300 × 1-L samples per region may be needed to adequately document fish diversity in species-rich tropical ecosystems like the Coral Triangle, but recognized that this may not be feasible and recommended 30 × 10-L samples as an alternative. This amount is still challenging when sampling in remote locations with limited resources for processing and filtering of samples in a timely manner to prevent sample deterioration. Here, collected only 1200 ml per anesthetic station so that we could more closely reflect the volume of the interstices where the CRF would be found and avoid diluting the sample with water from outside the reef matrix, and it is possible that restricting total volume may have prevented detecting more CRF. Nevertheless, other eDNA studies that have detected CRF using the COI locus have also had poor results when collecting larger volume samples either near the substrate or from the water column. When compared to the 4 CRFs detected from 13,900 ml of water in our study, Nguyen et al. (2020) detected 13 species of CRF in the Caribbean, after filtering 134,000 ml of water sampled just above the benthos (10–30 cm), while in Indonesia, Gelis et al. (2021) detected just one CRF species after filtering 112,000 ml of water collected from the water column and from substrate samples. Water volume seems to be an important factor affecting fish detections from eDNA samples and achieving a leveling of accumulation curves for species detected remains an elusive goal even for studies that filter copious volumes of water (Marwayana et al. 2021; Bessey et al. 2020).

Another important factor that may have limited our detection of CRF is the use of primers that target all eukaryotes (Leray 2013). This fact, combined with the collection of our samples from the substrate, possibly contributed to having excess amounts of marine metazoans other than CRF, which may have overwhelmed the low levels

of eDNA from CRF. Nguyen et al. (2020) targeted fishes and invertebrates using the COI primers from Leray et al. (2013). They recognized that the co-amplification of non-targeted metazoans, as well as non-metazoan taxa, which is common when using this loci/primer combination, may have lowered detection of rare taxa of fishes and invertebrates in their samples. We chose this locus and primer combination despite this, rather than using fish-specific primers for loci like 12S or 16S, since the latter genes have comparatively fewer sequences for CRF in reference databases and lack the sensitivity required to differentiate CRF at the species level.

One of the main obstacles echoed in many metabarcoding studies is the incomplete coverage of taxa in available public molecular databases, along with cases where sequences are assigned to the wrong species due to contamination or public sequences linked to misidentified voucher specimens. For example, our samples contained 2176 reads that according to GenBank were assigned to the ray-finned fish *Alburnus scoranza* (99–100% identity), a freshwater species only found in a European lake. When running that specific sequence as a separate search in BOLD, the result was *Homo sapiens* at a 100% match. Using the MARES + *Eviota* database for taxonomic matching after BLASTing the curated sequenced data revealed that except for *Eviota cf. punctulata* specimen sequences from Tonga that were recently uploaded to BOLD and GenBank from a recent publication (Greenfield et al. 2018), none of our eDNA sequences for CRF matched sequences from BOLD and GenBank. This confirms the underrepresentation of CRF in general in public genetic databases and may also be an indication of the underrepresentation of fish sequences in general from under sampled tropical regions (Nguyen et al. 2020).

The fact that so few CRF species are detected in eDNA studies may be related to their high levels of micro-allopatry and endemism (Brandl et al. 2018). Many groups of CRF, including some of the most diverse and abundant genera such as *Eviota*, speciate across extremely fine geographic scales (Tornabene et al. 2015; Taylor and Hellberg 2005; Victor 2015), which is likely the result of limited gene flow, local larval retention, and rapid evolutionary rates (Brandl et al. 2018, 2019). This results in species complexes containing morphologically similar species that are restricted to their own island or island group and differ genetically by as little as 0.5% divergence in COI in recently diverged species (Greenfield and Tornabene 2014), to > 5% divergence in older species others (Tornabene et al. 2016). Many of these micro-allopatric species have yet to be described (Winterbottom eta al. 2014, 2020, Greenfield 2017; Greenfield et al. 2018). This further emphasizes the need for comprehensive taxonomic studies on CRFs that span geographic regions, as well as DNA barcoding efforts that sample densely within geographic regions (Weigt et al. 2012; Victor 2015; Winterbottom et al. 2020).

Even though sampling of CRF has been done now in many regions (see Brandl et al. 2018 for a review), it was not until the early 2000s that most surveys included photographic records, tissue samples or cataloging of voucher specimens in collections. Using underwater visual surveys and from museum records of specimens collected in multiple expeditions, Randall et al. (2003) published an exhaustive checklist of fishes from the Tongan Archipelago from, reporting as many as 296 CRF species. Recent underwater visual surveys from a rapid biological survey conducted in the Vava'u Archipelago (Atherton et al. 2014) focused on fish species > 5 cm. Despite CRFs not being a targeted group, their checklist included two Gobiidae and two Blenniidae species. Nevertheless, our study, even if restricted to the Vava'u island group, is the first that we know in which the collection of CRF includes photographs, tissues, and voucher specimens from Tonga.

Conclusion

Future eDNA or metabarcoding efforts for documenting the community assemblages of CRF should go hand in hand with taxonomic efforts to robustly describe faunas and contribute DNA sequences to public databases. DNA sequences should be linked to specimens cataloged in collections available to the scientific community (de Santana et al. 2021). This is especially relevant for CRFs, which are an integral component of reef fish assemblages, but have mostly been ignored using traditional underwater survey methods. Although our study concludes that AS are the most reliable technique to study CRF community assemblages, and that eDNA sampling may not be suitable at this time to reliably document CRFs, we foresee a continued effort to refine eDNA outcomes, including improvements during collection of samples through the final steps in the data processing. These efforts should go hand in hand with collections of CRF specimens in under sampled regions, including rigorous taxonomic identifications and molecular analysis that will ultimately help document CRF biodiversity.

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Declaration

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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