DETECTING GROUPER (EPINEPHELINAE) DIET COMPOSITION AND PREY AVAILABILITY IN RAJA AMPAT CORAL REEFS THROUGH DNA METABARCODING

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ABSTRACT

Gaining extensive knowledge of prey sources is an essential approach for understanding trophic structure and relationships, especially in highly diverse coral reef ecosystems. *Epinephelus areolatus* and *E. malabaricus* have different characteristics based on niche selection, food items, and predatory behavior. This study was conducted to investigate and compare the taxonomic classification and composition of prey in the diets of two common species of groupers (*Epinephelus areolatus* and *E. malabaricus*) based on DNA metabarcoding of gut contents and potential prey detection using environmental DNA tools at sites in the coral reefs of Raja Ampat, Indonesia. Samples were collected by means of scuba diving and the use of spear guns. Water samples were filtered, while gut content samples were obtained by dissecting the groupers samples, before being taken to the laboratory for metabarcoding analysis. DNA recovered from the water column comprised taxa from the Classes Arthropoda, Chordata, Cnidaria and Mollusca, several of which were also found in grouper guts, with Cnidaria as the most abundant class. Diversity was high for potential prey species in the environment and prey consumed by each grouper species. The highly overlapping in prey identified from gut contents indicated that the two epinephelids have a similar feeding strategy. However, nMDS ordination showed segregation between the prey consumed by each species and potential prey available in the environment. The results indicated a low likelihood of competition between the two grouper species, related to the abundance and diverse choice of potential prey in the highly biodiverse Raja Ampat coral reef ecosystem.

Keywords: alpha diversity, environmental DNA, food web, gut content analysis, high throughput sequencing

INTRODUCTION

Reef fishes are in high demand as commercial, recreational, and ornamental commodities. In Asia, the reef fish trade is mostly dominated by groupers, especially the genus *Epinephelus*. Grouper production in Indonesia was recorded as 48,422,000 tonnes in 2012 (Yulianto *et al.* 2015a). Predatory fish such as groupers play crucial ecological roles, influencing or even regulating the behavior (Roff *et al.* 2016) and abundance (Stier & White 2014) of their prey.

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The commercial fishing pressures on groupers and their broad distributions, especially in eastern Indonesia, can make them as appropriate models for investigating species-specific predator-prey relations in reef fishes (Jefri *et al.* 2015; Yulianto *et al.* 2015b, a; Luiz *et al.* 2016). In Raja Ampat, Indonesia, local fishermen apply Sasi Laut as a form of mitigation against anthropogenic pressures. This local wisdom can enable effective conservation and maintain the structure of marine communities, and can thereby help maintain the sustainability of grouper fisheries (Harkes & Novaczek 2002; McLeod *et al.* 2009). Therefore, Raja Ampat offers opportunities for

investigating predator-prey relationships in coral reef communities with relatively low direct human impacts.

Grouper predation can be influenced in terms of both behavior and food composition by their habitats and environmental conditions, such as niche availability, prey dynamics, and anthropogenic pressures (Kline *et al.* 2011; Mahjoub *et al.* 2011; Jo *et al.* 2013; Moran *et al.* 2015; Diopere *et al.* 2018; Starr *et al.* 2018). Several studies on grouper feeding habits (prey) using visual gut content examination methods provided useful yet incomplete information (Leray *et al.* 2019), as much of the gut content is generally unrecognizable (Berg 1979; Ruppert *et al.* 2019) and findings are rarely related to comprehensive and timely data regarding the prey availability in the surrounding environment (Baker *et al.* 2014).

Metabarcoding can detect DNA present in degraded matter, such as much of fish gut content (Harms-Tuohy *et al.* 2016), while environmental DNA (eDNA) methods can provide information related to the inhabitants of coral reef ecosystems, especially in complex biotic communities (Thomsen *et al.* 2012; Yamanaka & Minamoto 2016).

This study investigated predator-prey relationships of two common groupers in Raja Ampat coral reefs (*Epinephelus areolatus* and *E. malabaricus*) through DNA metabarcoding of gut contents (actual prey composition samples) and environmental DNA (eDNA) metabarcoding of the water column (potential prey availability). The aim of using these genetic tools was to acquire in-depth information on grouper diets in terms of species composition, to estimate relative abundance and the potential for inter-species competition, and to evaluate the relation between observed diets and prey availability. These data will inform the management of two important grouper species and serve as a basis for future research on trophic relations in reef fish communities.

MATERIALS AND METHODS

Sample Collection and Processing on the Field

This research was conducted in Raja Ampat, Indonesia with a focus on Cape Kri and Arborek (Fig. 1). On-site sampling was performed during the dry and transitional seasons, in order to avoid rough sea conditions (Fahlevy *et al.* 2019; Prabowo *et al.* 2019).

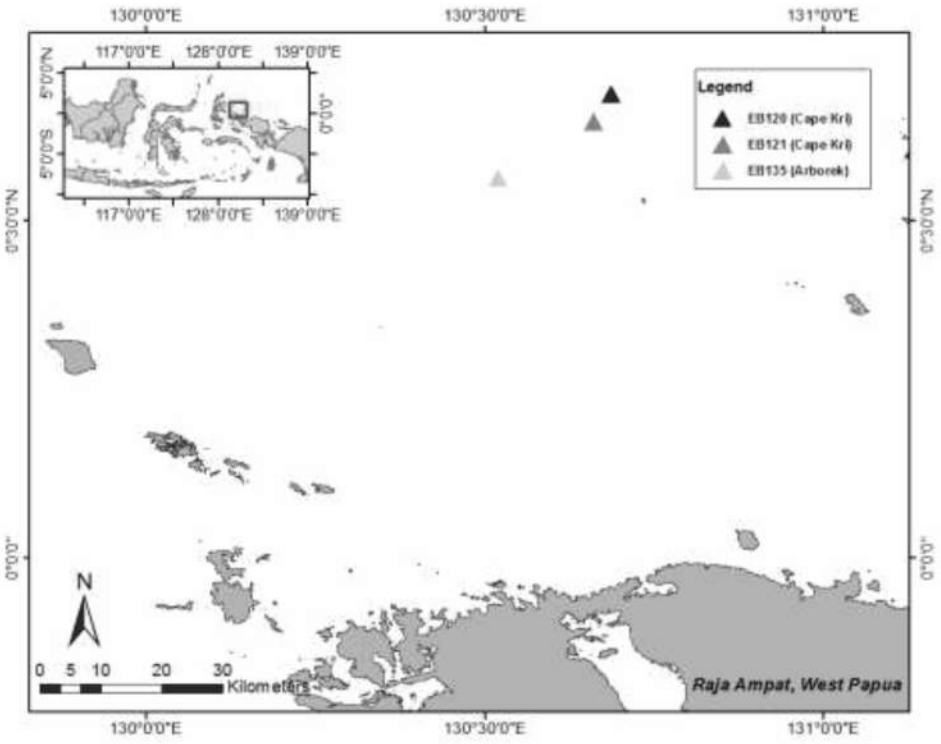

Figure 1 Location of Cape Kri and Arborek in Raja Ampat, the two study sites having different coral reef ecosystem typology

Specimens of the two most common grouper species in Raja Ampat, *Epinephelus areolatus* and *Epinephelus malabaricus*, were collected from each site by fishermen using spear guns, and processed immediately after capture in fresh condition. The fish were immediately dissected to remove the guts to avoid post-mortality voiding and decomposition. All preyed organisms were removed from the gut content. After removing the preyed organisms, the gut contents from each sample were ground and placed in 5 mL cryotubes pre-filled with 4 mL ZymoBIOMICS DNA/RNA shield (Zymo Research Corp. USA).

Seawater samples were collected directly from the water column near the substrate to represent the coral reef ecosystem biotic community. Divers using Self-Contained Underwater Breathing Apparatus (SCUBA) equipment collected the water in 4 L bottles and brought the water samples to the surface where each sample was then filtered through a 0.4 µm sterilized Pall filter membrane (Pall Corporation, USA) using a peristaltic pump. Each filter membrane was placed in a 2 mL cryotube pre-filled with 1 mL ZymoBIOMICS DNA/RNA shield (Zymo Research Corp. USA). Contamination was prevented through the sterilization of all the sampling equipment used at each stage of the sampling procedure with a 10% solution of commercial bleach.

Molecular Analysis

Fish gut content and eDNA samples were extracted using Qiagen DNeasy Blood and Tissue Kits following the manufacturer's manual instruction. Polymerase Chain Reaction (PCR) was conducted to obtain partial gene of Cytochrome C oxidase subunit I (CO1) mitochondrial DNA (mtDNA) fragments. The targeted fragment of 313 bp were amplified using the universal primer pair dgHCO2198 (5' - TAA ACT TCA GGG TGA CCA AAR AAY CA – 3') and mICOIintF (5' - GGW ACW GGW TGA ACW GTW TAY CCY CC – 3') to reveal mock community of metazoan (Meyer 2003; Leray *et al.* 2013).

The PCR reaction comprised 0.6 µL each of 10 µM forward and reverse universal primers (dgHCO2198 and mICOIintF), 0.2 µL Biolase taq polymerase (Bioline) 5 U.µL-1, 0.8 µL of 50 mM MG2+, 1 µL of 10 µM dNTP and 1 µL of DNA template. Thermocycle was performed by following initial denaturation for 5 minutes at 95 °C and followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing for 45 seconds at 48 °C, and extension for 30 seconds at 72 °C, with final extension at 72 °C for 10 minutes (Leray *et al.* 2016). The PCR product was then checked using 2% gel electrophoresis.

The amplicon products were then prepared to Next Generation Sequencing by following Illumina procedure. The dual indices and Illumina sequencing adapters from the IDT for Illumina - Nextera DNA Unique Dual Indexes, Set A (catalog number 20027213) (Illumina, San Diego, USA) were added to the target amplicons in a second PCR step using Kapa HotStart HiFi 2× ReadyMix DNA polymerase (Kapa Biosystems Ltd., London, UK). Cycle conditions were 95 °C (3 minutes), then 9 cycles of 95 °C (30 seconds), 55 °C (30 seconds), 72 °C (30 seconds), then a final extension of 72 °C (5 minutes).

Libraries were quantified on a fluorometric Qubit. The barcoded amplicon libraries were combined in equal concentrations into a single pool according to their quantification measurement. The library pool was diluted and denatured according to the Illumina MiSeq library preparation guide. The amplicon library (10 pM) was spiked with 20% denatured and diluted PhiX Illumina control library version 3. The sequencing was conducted on the Illumina MiSeq using the MiSeq reagent kit V3 600 cycle at the University of Rhode Island, Kingston, USA.

Bioinformatics Pipeline

Sequence data obtained were trimmed using Cutadapt software (Martin 2011) to remove the unique dual Illumina indexes and primer sequences. All sequences were then filtered, merged, and de-noised using DADA2 pipeline (Callahan 2016). DADA2 is the commonly used pipeline in amplicon sequencing study with the Next Generation Sequencing method to produce Amplicon Sequence Variance (ASV).

The ASV was then imported to MEGA X software (Kumar *et al.* 2018) as a Fasta file to identify each sequence through the NCBI BLAST (https://www.ncbi.nlm. nih.gov/). The

organism blasting options used excluded "insecta" from the sequence identification process. Several marine arthropods were identified as terrestrial arthropod species when this option was not applied.

Highly similar sequences obtained from the BLAST algorithm were used for identification to the nearest match among the taxa in the database. Each sequence in the final database produced represented one individual species/ taxon.

Ecological Statistical Analysis

Individual and sample-based rarefaction curves were produced to assess the effect of sequencing effort. This method works by separately calculating many curves using random samples and data reads at increasing degrees of accumulation and was implemented in EstimateS software (Colwell 2013). Sequencing reads were used to determine the relative abundance of identified species (Lacoursière-Roussel *et al.* 2016; Pont *et al.* 2018).

Multivariate environmental analysis was performed in PRIMER 7 (Kruskal 1964; Clarke & Gorley 2015) to examine the composition of food (prey) items found in the environment and in grouper guts. A nonmetric multidimensional scaling (nMDS) comparison of taxa present in gut content and the environment were developed from the Bray-Curtis similarity index. This nMDS analysis was performed to visualize patterns of food items from each source using quadratic root transformation. This transformation was used to reduce the discrepancy between the abundant and the uncommon species by down-weighting abundant species relative to uncommon species (Clarke 1993). Venn diagrams were built using InteractiVenn (Heberle *et al.* 2015) to identify similar species found in different data sets, even those with little intersection shown in the nMDS analysis.

Differences in food item composition between the two species were evaluated from the gut content and eDNA data using PERMANOVA with 1,000 permutations to test the robustness of the ecological patterns from the nMDS analysis (Anderson & Walsh 2013; Ruppert *et al.* 2017). The most abundant or predominant food items were determined using Similarity Percentage (SIMPER) analysis. This method provides information on the characteristics and the relationships between sampled items (Leray *et al.* 2019). The gut content data for each of the two grouper species were then examined separately using SIMPER with a 50% cut-off.

RESULTS AND DISCUSSION

Diversity and Abundance of Prey Items from Gut Content and Environmental DNA

Out of 27,000 reads trimmed and validated by the de-noising process, the MEGA BLAST routine matched 26,100 sequences with barcode accessions in the GenBank or BOLD databases, of which 11,584 identified as *Epinephelus areolatus* or *Epinephelus malabaricus*, the fish from which the gut specimens were collected. There were 517 reads representing contamination or microbes (*Homo sapiens*, bacteria, proteobacteria, etc.) and 383 unidentified reads (Fig. 2a).

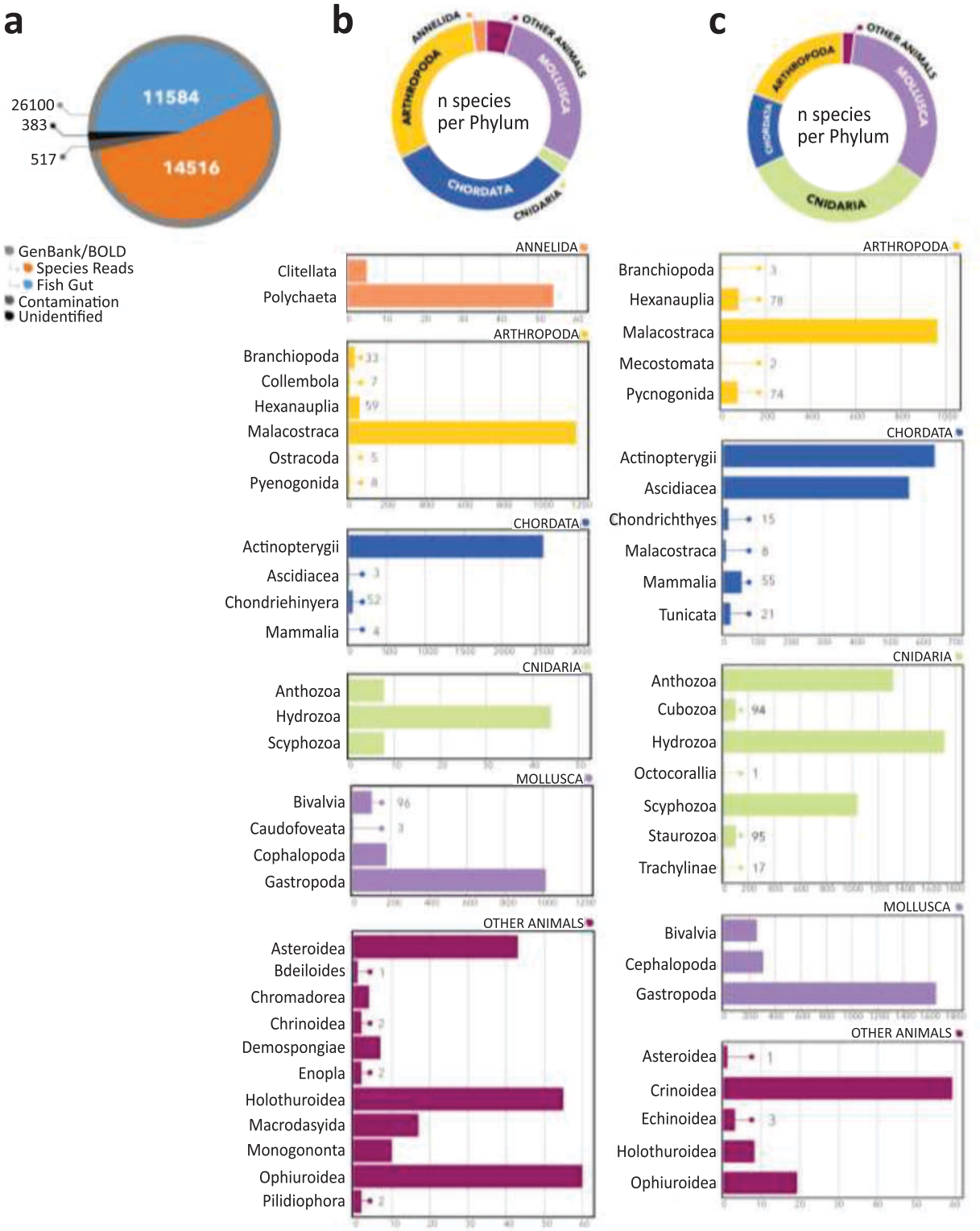

Figure 2 Diversity of taxa identified from CO1 metabarcoding

Notes: (a) DNA sequence reads obtained from *Epinephelus areolatus* (n = 3) and *E. malabaricus* (n = 3) gut contents and eDNA (n = 3); (b) taxa identified from gut contents; and (c) taxa identified from eDNA. The "Other animals" group included Echinodermata, Rotifera, Porifera, Nemertea, Nematoda, and Gastrotricha.

The identified reads were identified as taxa belonging to 15 phyla, of which 15 phyla were found in the gut content samples and 5 in the eDNA samples. The phylum with the most reads was Chordata (2,601 reads, 264 species), followed by Mollusca (2,227 reads, 236 species), and Arthropoda (1,304 reads, 248 species). Annelida, Cnidaria, and other phyla comprised less than 5% of the gut content reads. In the eDNA samples, Cnidaria was the phylum with the highest number of reads (4,265 reads, 213 species), followed by Chordata (1,298 reads, 83 species), Mollusca (1,233 reads, 209 species), and Arthropoda (1,120 reads, 123 species).

The prey items identified from the gut contents of both *E. areolatus* and *E. malabaricus* belonging to the phylum Chordata were mostly fishes (Fig. 2b) from several families. Phylum Arthropoda was the second most preferred grouper's prey group, comprising crabs and shrimps as well as members of the Classes Amphipoda and Isopoda. Prey from the phylum Mollusca comprised bivalves, gastropods (including nudibranchs) and cephalopods, such as squids and octopuses. The phylum Annelida was present with low read numbers in *E. areolatus* gut samples and some Cnidaria were present in *E. malabaricus* gut samples.

The potential prey identified in eDNA samples were similar to those identified from the grouper gut content samples (Fig. 2c). The main difference was the abundance of jellyfish (phylum Cnidaria). The reads from eDNA samples (collected from the water column) were mostly identified as slow or passive moving organisms, such as phytoplankton, zooplankton, and jellyfish (Harvey & Menden-Deuer 2012; van Elden *et al.* 2014; Nath *et al.* 2017). The identified Mollusca from the eDNA samples included shellfish (bivalves and gastropods), nudibranchs, squid, and octopus, while the Chordata comprised fishes and tunicates, and Arthropods included crabs (e.g., blue swimming crabs) and shrimps.

Rarefaction curves produced for individual samples and aggregated data (Fig. 3a) showed a flattening of the curves at around 2,000 to 2,500 total reads, which indicated that the sequencing data were sufficiently representative. The same as the individual-based rarefaction analysis, the intercalation of samples resulted in the increasing of sequences reads (Fig. 3b). The rarefaction analysis indicated that the number of samples used was sufficient to produce representative sequence data. Therefore, the data obtained from the gut content and eDNA samples can be considered adequate and sufficient to illustrate the diversity of grouper food items consumed, as well as the potential prey items present in Raja Ampat coral reefs ecosystems and to enable meaningful comparisons between them.

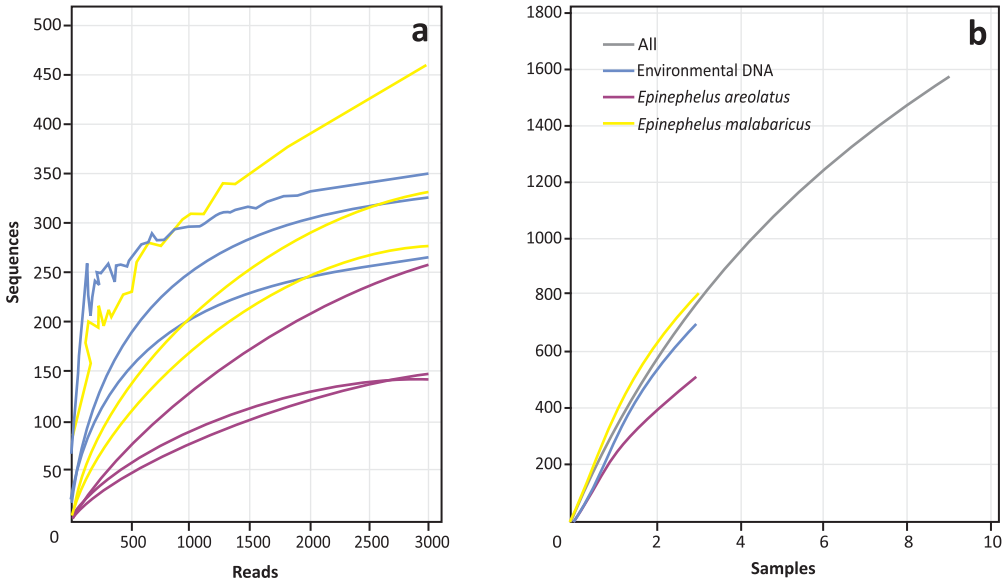

Figure 3 Rarefaction curves representing sequencing depth based on random re-sampling of sequences for: (a) individual samples and (b) the three sample types and all samples combined
Note: the rarefaction curves indicate that the sequencing was adequate to describe the diversity of grouper gut contents.

The relationship between each species and their food items illustrates the mechanisms and dietary character of coral reef fishes, and also creates high predation network, due to the level of diversity (Veron *et al.* 2009; Grol *et al.* 2011; 2014; Miller *et al.* 2016). The lack of food items shown by taxonomic methods and high potential food resources in coral reefs, aid in identifying and quantifying their dietary differences. This study showed that molecular analysis using metabarcoding methods can provide a substantial amount of information, especially for groupers with a high diversity of food items. Focused on the dietary composition of two grouper species, the study was able to access a large metazoan sequence database to identify taxa present within their guts and, through the DNA traces left in the water column, in their habitat. Overall, 1,428 species were identified, belonging to 42 classes and 15 phyla.

The *Epinephelus* grouper food items were highly diverse, including not only nekton, such as smaller fishes, but also small cryptic species that live between corals and other benthic substrate, such as crabs, shrimps, and nudibranchs. Based on reads proportion, the moon crab *Matuta planipes* was the most abundant crustacean found in the guts of both groupers, followed by the blue swimming crab *Portunus sanguinolentus*. Moderate-sized demersal invertebrates are considered as potential grouper prey (Koenig *et al.* 2011; Artero *et al.* 2015). As top predators, grouper predation patterns generally involve waiting for prey to approach their territory before chasing them (Luckhurst 2010; Bessa 2011).

Groupers are also known as territorial fishes that tend to dwell in crevices within the coral reef structure (Farmer & Ault 2011; Wall *et al.* 2011; Koeck *et al.* 2014). Examples of grouper prey that can be found in reef crevices include *Hippocampus pontohi*, a species of sea horse. Gobiidae was found to be the most dominant fish family, and possessed the behavior of staying on substrates, with minimal movement compared to other fish families (Choi & Suk 2012; Bo *et al.* 2014; Teichert *et al.* 2014; Laramie *et al.* 2015). This indicated that the groupers do not wander distantly from its territory. The moderate-sized reef fishes were also found as groupers prey, such as those from families Acanthuridae, Apogonidae, Carangidae, Scaridae, and Siganidae. The variety of fish taxa found in grouper guts confirms the high biodiversity, especially for fishes, reported from visual surveys of coral reefs in Raja Ampat (Veron *et al.* 2009; Bachtiar *et al.* 2011; Carpenter *et al.* 2011).

Groupers can feed on different prey in different regions. For example, groupers in Brazil and Tanzania mostly feed on molluscs, especially nektonic cephalopods, such as octopus, squid, and moderate-size cuttlefish (Condini *et al.* 2015; Gaspare *et al.* 2015). The variety of species consumed by groupers from different taxa can also be influenced by the feeding behavior and morphology of each species (Wainwright *et al.* 1995; St John 1999). In this study, the two carnivorous groupers could readily coexist as, despite an overlap in prey species, they each consumes different groups of species available in the coral reef environment. Although there was an overlap of 238 taxa in the gut contents of the two grouper species, these comprised mostly species with low biomass and non-prey items.

Proximity Patterns between Grouper Diet Composition and Food Availability in the Environment

The nMDS represents the food items between the three sample groups (Fig. 4). However, there was one similarity between *Epinephelus areolatus* and environmental DNA, which had narrow differences with *Epinephelus malabaricus*. The pattern seen in the nMDS plot was well supported by the PERMANOVA statistical tests, with significant differences between the two grouper species and between the sample types (grouper gut content and eDNA) (Table 1).

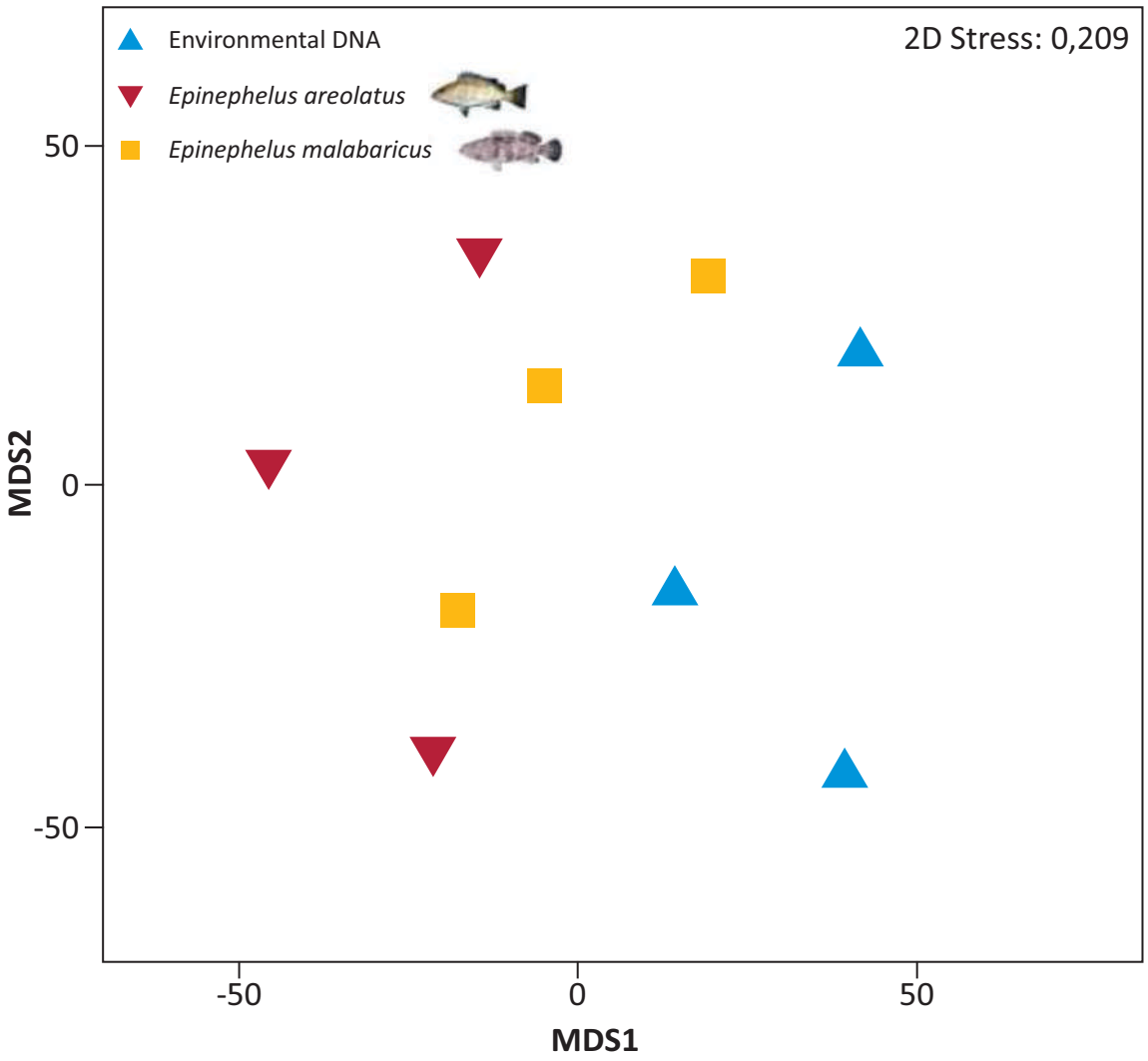

Figure 4 Non-metric Multi Dimensional Scaling (nMDS) ordination plot comparing the composition of identified reads from grouper gut contents and the surrounding environment (eDNA)

Table 1 Permutational multivariate analysis (PERMANOVA) analysis of differences in composition between (inter-specific) and within (intra-specific) taxa identified through metabarcoding gut contents of two grouper species (*E. areolatus* and *E. malabaricus*)

	Source of variation	df	SS	F Model	R^2	P value
Intra-specific	Individual fish	2	12113	45,278	0.1	0.005
	Residuals	6	8025.6		0.9	
Inter-specific (Raw Data Type)	Species	1	9876.6	67,372	0.22	0.012
	Residuals	7	10262		0.78	

The nMDS ordination plot did to show clear grouping patterns, however, the Venn diagram based on species composition showed overlaps in the taxa identified from each sample type (Fig. 5). Collectively, 63 taxa identified in the surrounding environment (eDNA samples) were also detected in the guts of one or both grouper species, while 238 species (of which only 27 were detected in the environment) were found in the guts of both grouper species. The majority of the taxa identified through environmental DNA metabarcoding (90%, 577 species) were not consumed by any of the sampled *Epinephelus* groupers. Conversely, the majority of taxa identified in grouper guts (92.6%, 788) were not identified from the eDNA samples. Of the 851 taxa found in grouper guts, 28% were identified from both grouper species, 22% only from *E. areolatus* and 50% only from *E. malabaricus*.

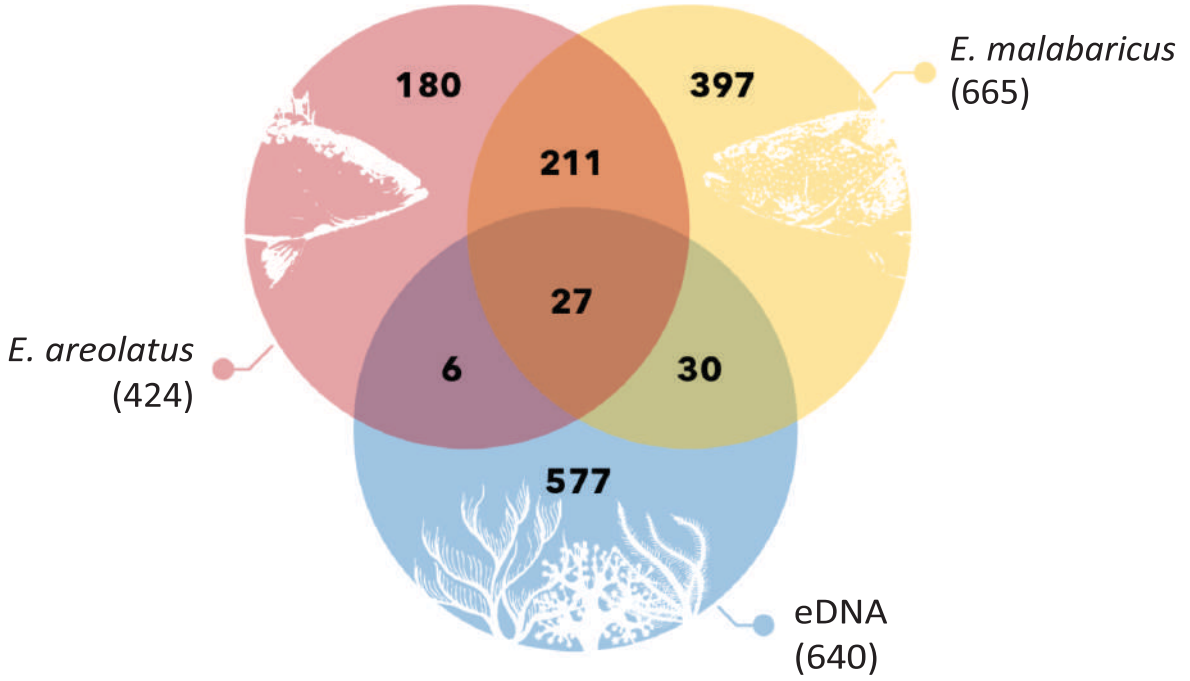

Figure 5 Venn diagram showing the overlap in taxa identified from metabarcoding the gut contents of *Epinephelus areolatus* (n = 3) and *E. malabaricus* (n = 3) and environmental DNA (n = 3), from Raja Ampat reefs

The phyla contributing the most species were Chordata, Arthropoda, and Mollusca. From the Chordata, *Cottoperco gobio* contributed the highest number of reads. Another grouper species, *Epinephelus fuscoguttatus*, was also one of the most abundant taxa, followed by other bony fishes. Reads of molluscan taxa were mostly shellfish, from *E. malabaricus* gut and eDNA samples. Crabs, including blue swimming crabs, contributed the most reads from the phylum Arthropoda, with jellyfish making the highest contribution to cnidarian reads (Table 2).

Table 2 Taxa which highly contribute to Similarity Percentage (SIMPER) pairwise comparison differences

Phylum	Lowest taxon	Contribution (%) to difference		
		E. areolatus versus *E. malabaricus*	*E. areolatus* versus eDNA	*E. malabaricus* versus eDNA
Chordata	*Cottoperca gobio*	*7.91	#0.14	*3.09
Chordata	*Epinephelus fuscoguttatus*	*4.3	#0.45	*1.8
Chordata	*Lateolabrax maculatus*	*4.1	x0.12	*1.29
Chordata	*Sparus aurata*	*3.61	x0.23	*0.86
Chordata	*Myripristis murdjan*	*3.48	#0.36	*0.62
Mollusca	*Conus stupa*	*2.4	0	*0.64
Mollusca	*Conus litoglyphus*	*2.39	x0.19	*0.54
Arthropoda	*Matuta planipes*	#1.69	#1.34	*0.42
Arthropoda	*Portunus sanguinolentus*	*1.46	0	0
Chordata	*Echeneis naucrates*	*1.34	#0.11	0
Chordata	*Ascidia ahodori*	0	x4.13	x3.46
Mollusca	*Sulcospira paludiformis*	0	x3.94	x3.29
Cnidaria	*Oulastrea crispata*	0	x3.3	x2.76
Cnidaria	*Caryophyllia smithii*	0	x2.91	x2.43
Arthropoda	*Munida taenia*	#0.27	x1.98	x1.02
Cnidaria	*Clytia gracilis*	0	x1.84	x1.74
Cnidaria	*Chrysaora melanaster*	#0.09	x1.78	x1.54
Mollusca	*Kurtiella bidentata*	0	x1.48	x1.23
Cnidaria	*Chrysaora pacifica*	*0.09	x1.22	x0.95

Notes: The sample type with the highest relative abundance (%) for each comparison is indicated by the symbols: # = *E. areolatus;* * = *E. malabaricus;* x = eDNA.

The Venn diagram shows considerable differences between the diets of each grouper species and the potential prey available in the environment (Fig 5), but there is no clear group segregation in the nMDS ordination (Fig. 4). The high diversity of prey species in the environment is the most likely explanation for the lack of clustering in the nMDS diagram.

A coral reef environment that provides a diverse and abundant diet is likely to result in high intra-species (individual) variation in diet, as well as (and potentially confounding) between-species differences. This indicates that competition for food in these two species is likely to be low, at least in such high quality habitat.

Groupers share a similar feeding mechanism, engulfing prey by means of their extensible jaws, which enable them to greatly expand their oral cavity (Kohno *et al.* 1997; Dierking *et al.* 2009; Collins & Motta 2017). It can take several hours for groupers to swallow and digest larger prey, such as moderate-sized reef fishes (Hseu *et al.* 2007). Both species in this study are reported to be ambush predators employing suction feeding (Randall 1967; Gibran 2007; Artero *et al.* 2015). Based on the nekton taxa found in gut content samples, both groupers can chase pelagic prey within the water column, as reported by Oufiero *et al.* (2012) and Collins and Motta (2017).

Groupers have also been found to have different feeding patterns, depending on the environmental conditions of the coral reefs where they live (Frisch *et al.* 2016). Environmental DNA taken from the water column near coral reefs can represent the types of food available. The Cnidaria, especially jellyfish, are one of the most dominant potential food sources generally found in the water column (Ki *et al.* 2010; Lucas *et al.* 2016). Pelagic cnidarians mostly use their tentacles to move (Straehler-Pohl & Jarms 2011).

Coral species can also be found in eDNA, as in this study. Other studies using eDNA metabarcoding have succeeded in detecting a high proportion of species present (Alexander *et al.* 2020; Holman *et al.* 2019; West *et al.* 2020). Taxa recovered from the eDNA and also found in the grouper gut samples included arthropods, chordates and molluscs. However, read abundances in the eDNA samples were lower for these taxa than for the cnidarians.

Some results from this study could potentially indicated the presence of species originating from outside the Indonesian region, based on the reads obtained from both the grouper gut contents and the eDNA samples. One of these species is *Cottoperca gobio*, a goby with a reported distribution around Chile and Argentina (Froese & Pauly 2000). Others include *Lateolabrax maculatus*, with a reported distribution in Japanese waters (Froese & Pauly 2000) and *Sparus aurata* from New Zealand (Froese & Pauly 2000).

One reason that such cases can occur in metabarcoding-based research because the metabarcoding method matches relatively short DNA sequences with the data contained in GenBank, sometimes resulting in spurious matches (Thomsen *et al.* 2012; Jo *et al.* 2013; Leray *et al.* 2016, 2019). Based on the unidentified sequence reads, it is clear that there are taxa for which no reference sequences are available, indicating that the genetic data in GenBank and BOLD repositories are still incomplete, especially for highly biodiverse areas such as the reefs of Raja Ampat.

Those three non-native fish species are morphologically and taxonomically similar to fishes that live in the waters of Raja Ampat. One possibility is that, due to the lack of genetic information for some organisms living in the waters of Raja Ampat, their sequences were most closely matched with those of other species deposited in the GenBank database. This study also showed that eDNA can identify several cryptic species that take shelter in coral reefs and are considered as grouper prey.

CONCLUSION

The identified reads were identified as taxa belonging to 15 phyla, of which 15 phyla were found in the gut content samples and 5 in the eDNA samples. The phylum with the most reads was Chordata with 2,601 sequences representing 264 species, followed by Mollusca (2,227 reads, 236 species), and Arthropoda (1,304 reads, 248 species). Annelida, Cnidaria, and other phyla comprised less than 5% of the gut content reads. In the eDNA samples, Cnidaria was the phylum with the highest number of reads (4,265) and species (213), followed by Chordata (1,298 reads, 83 species),

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REFERENCES

Alexander JB, Bunce M, White N, Wilkinson SP, Adam AA, Berry T, …, Richards ZT. 2020. Development of a multi-assay approach for monitoring coral diversity using eDNA metabarcoding. Coral Reefs 39(1): 159-71.

Anderson MJ, Walsh DCI. 2013. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? Ecol Monogr 83: 557-74. DOI: 10.1890/12-2010.1

Artero C, Koenig C, Richard P, Berzins R, Guillou G, Bouchon C, Lampert L. 2015. Ontogenetic dietary and habitat shifts in goliath grouper *Epinephelus itajara* from French Guiana. Endanger Species Res 27: 155-68. DOI: 10.3354/esr00661

Bachtiar I, Damar A, Suharsono, Zamani NP. 2011. Assessing ecological resilience of Indonesian coral reefs. J Coast Dev 3: 214-22 .

Baker R, Buckland A, Sheaves M. 2014. Fish gut content analysis: Robust measures of diet composition. Fish Fish 15(1): 170-7.

Berg J. 1979. Discussion of methods of investigating the food of fishes, with reference to a preliminary study of the prey of *Gobiusculus flavescens* (Gobiidae). Mar Biol 50(3): 263-73.

Bessa E. 2011. The fitness of the Brazilian damsel *Stegastes fuscus* is increased by sharing the territory with the dusky grouper *Epinephelus marginatus*. Acta Ethol 14: 97-102. DOI: 10.1007/s10211-011-0094-9

Bo T, López-rodríguez MJ, Fenoglio S, Cammarata M, de Figueroa JMT. 2014. Feeding habits of *Padogobius bonelli* (Osteichthyes: Gobiidae) in the Curone Creek (Northwest Italy): Territoriality Influences Diet? J Freshw Ecol 25: 367-71. DOI: 10.1080/02705060.2010. 9664379

Bosley KL, Miller TW, Brodeur RD, Bosley KM, Gaest AV, Elz A. 2014. Feeding ecology of juvenile rockfishes off Oregon and Washington based on gut content and stable isotope analyses. Mar Biol 161: 2381-93. DOI: 10.1007/s00227-014-2513-8

Brandl SJ, Robbins WD, Bellwood DR, Brandl SJ. 2015. Exploring the nature of ecological specialization in a coral reef fish community: Morphology, diet and foraging microhabitat use. Proc R Soc B Biol Sci 282: 20151147.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 13(7): 581-3.

Carpenter KE, Barber PH, Crandall ED, Ablan-Lagman MCA, Ambariyanto, Mahardika GN, …, Toha AHA. 2011. Comparative phylogeography of the coral triangle and implications for marine management. J Mar Biol (2011): 1-14. DOI: 10.1155/2011/396982

Choi S, Suk HY. 2012. The mechanisms leading to ontogenetic diet shift in a microcanivore, *Pterogobius elapoides* (Gobiidae). Animal Cells Syst (Seoul) 16: 343-9. DOI: 10.1080/19768354.2012.667002

Clarke KR. 1993. Non-parametric multivariate analyses of changes in community structure. Aust J Ecol 18: 117-43. DOI: 10.1111/j.1442-9993.1993.tb00438.x

Clarke KR, Gorley RN. 2015. PRIMER v7: User Manual and Tutorial, 1st Edition. Plymouth (UK): Primer-E Ltd.

Collins AB, Motta PJ. 2017. A kinematic investigation into the feeding behavior of the Goliath grouper *Epinephelus itajara*. Environ Biol Fishes 100: 309-23. DOI: 10.1007/s10641-016-0543-4

Colwell RK. 2013. EstimateS: Statistical estimation of species richness and shared species from samples. Version 9. User's Guide and application published at: http://purl.oclc.org/estimates.

Condini MV, Hoeinghaus DJ, Garcia AM. 2015. Trophic ecology of dusky grouper *Epinephelus marginatus* (Actinopterygii, Epinephelidae) in littoral and neritic habitats of southern Brazil as elucidated by gut contents and stable isotope analyses. Hydrobiologia 743: 109-25. DOI: 10.1007/s10750-014-2016-0

Dierking J, Williams ID, Walsh WJ. 2009. Diet composition and prey selection of the introduced grouper species peacock hind (*Cephalopholis argus*) in Hawaii. Fish Bull 107: 464-76.

Diopere E, Vandamme SG, Hablutzel PI, Cariani A, Houdt JV, Rijnsdorp A, …, Maes GE. 2018. Original article seascape genetics of a flatfish reveals local selection under high levels of gene flow. ICES J Mar Sci 75: 675-89. DOI: 10.1093/icesjms/fsx160

Donelson JM, Munday PL, McCormick MI, Pankhurst NW, Pankhurst PM. 2010. Effects of elevated water temperature and food availability on the reproductive performance of a coral reef fish. Mar Ecol Prog Ser 401: 233-43. DOI: 10.3354/meps08366

Fahlevy K, Prabowo B, Mubarok MWI, Fahrezi FY, Abdurrahman MI, Prasetia MF, …, Madduppa H. 2019. Comparing hard coral cover between Panggang and Kelapa Island Administrative Village, Seribu Islands National Park, Indonesia. IOP Conf Ser Earth Environ Sci 241: 012036. DOI: 10.1088/1755-1315/241/1/012036

Farmer NA, Ault JS. 2011. Grouper and snapper movements and habitat use in Dry Tortugas, Florida. Mar Ecol Prog Ser 433: 169-84. DOI: 10.3354/meps09198

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metozoan invertebrates. Mol Mar Biol Biotechnol 3: 294-9.

Freitas M, Abilhoa V, Giglio VJ, Hostim-Silva M, de Moura RL, Francini-Filho RB, Minte-Vera CV. 2015. Diet and reproduction of the goliath grouper, *Epinephelus itajara* (Actinopterygii: Perciformes: Serranidae), in Eastern Brazil. Acta Ichthyol Piscat 45: 1-11. DOI: 10.3750/AIP2015.45.1.01

Frisch AJ, Cameron DS, Pratchett MS, Williamson DH, Williams AJ, Reynolds AD, Hoey AS, Rizzari JR, Evans L, Kerrigan B, Muldoon G. 2016. Key aspects of the biology, fisheries and management of coral grouper. Rev Fish Biol Fish 26(3): 303-25.

Froese R, Pauly D (Editors). 2000. FishBase 2000: Concepts designs and data sources. ICLARM. Los Banos (PH): WorldFish. 344 p.

Gaspare L, Bryceson I, Kulindwa K. 2015. Ocean and coastal management complementarity of fishers' traditional ecological knowledge and conventional science: Contributions to the management of groupers (Epinephelinae) fisheries around Mafia Island, Tanzania. Ocean Coast Manag 114: 88-101. DOI: 10.1016/j.ocecoaman.2015.06.011

Gibran FZ. 2007. Activity, habitat use, feeding behavior, and diet of four sympatric species of Serranidae (Actinopterygii: Perciformes) in southeastern Brazil. Neotrop Ichthyol 5: 387-98. DOI: 10.1590/s1679-62252007000300018

Green SJ, Co IM. 2014. Trait-based diet selection: prey behavior and morphology predict vulnerability to predation in reef fish communities. J Anim Ecol 83: 1451-60. DOI: 10.1111/1365-2656.12250

Grol MGG, Nagelkerken I, Rypel AL, Layman CA. 2011. Simple ecological trade-offs give rise to emergent cross-ecosystem distributions of a coral reef fish. Oecologia 165: 79-88. DOI: 10.1007/s00442-010-1833-8

Grol MGG, Rypel AL, Nagelkerken I. 2014. Growth potential and predation risk drive ontogenetic shifts among nursery habitats in a coral reef fish. Mar Ecol Prog Ser 502: 229-44. DOI: 10.3354/meps10682

Harkes I, Novaczek I. 2002. Presence, performance, and institutional resilience of sasi, a traditional management institution in Central Maluku, Indonesia. Ocean Coast Manag 45: 237-60. DOI: 10.1016/S0964-5691(02)00057-1

Harms-Tuohy CA, Schizas NV, Appeldoorn RS. 2016. Use of DNA metabarcoding for gut content analysis in the invasive lionfish *Pterois volitans* in Puerto Rico. Mar Ecol Prog Ser 558: 181-91.

Harvey EL, Menden-Deuer S. 2012. Predator-induced fleeing behaviors in phytoplankton: A new mechanism for harmful algal bloom formation? PLoS One 7: e46438. DOI: 10.1371/journal.pone.0046438

Heatwole SJ, Fulton CJ. 2013. Behavioral flexibility in reef fishes responding to a rapidly changing wave environment. Mar Biol 160: 677-89. DOI: 10.1007/s00227-012-2123-2

Heberle H, Meirelles GV, Silva FR, Telles GP, Minghim R. 2015. InteractiVenn: A web-based tool for the analysis of sets through Venn diagrams. BMC Bioinformatics 16: 1-7. DOI: 10.1186/s12859-015-0611-3

Holman LE, Hollenbeck CM, Ashton TJ, Johnston IA. 2019. Demonstration of the use of environmental DNA for the non-invasive genotyping of a bivalve mollusk, the European flat oyster (*Ostrea edulis*). Front Genet 10: 1159.

Hseu J, Huang W, Chu Y. 2007. What causes cannibalization-associated suffocation in cultured brown-marbled grouper, *Epinephelus fuscoguttatus* (Forsskal, 1775)? Aquac Res 38: 1056-60. DOI: 10.1111/j.1365-2109.2007.01762.x

Hynes HB. 1950. The food of fresh-water sticklebacks (*Gasterosteus aculeatus* and *Pygosteus pungitius*), with a review of methods used in studies of the food of fishes. J Anim Ecol 1: 36-58.

Jefri E, Zamani NP, Subhan B, Madduppa H. 2015. Molecular phylogeny inferred from mitochondrial DNA of the grouper *Epinephelus* spp. in Indonesia collected from local fish market Tissue sampling. Biodiversitas 16: 254-63. DOI: 10.13057/biodiv/d160221

Jo H, Gim J, Jeong K, Kim HS, Joo G. 2013. Application of DNA barcoding for identification of freshwater carnivorous fish diets: Is number of prey items dependent on size class for *Micropterus salmoides*? Ecol Evol 4: 219-29. DOI: 10.1002/ece3.921

Jones GP. 1986. Food availability affects growth in a coral reef fish. Oecologia 70: 136-9. DOI: 10.1007/BF00377123

Ki J, Hwang D, Lee J. 2010. Simultaneous detection of *Aurelia* and *Chrysaora scyphozoan* jellyfish on a

DNA microarray. J Mar Biol Assoc United Kingdom 90: 1111-7. DOI: 10.1017/S0025315409990373

Kline RJ, Khan IA, Holt GJ. 2011. Behavior, color change and time for sexual inversion in the protogynous grouper (*Epinephelus adscensionis*). PLoS One 6: e19576. DOI: 10.1371/journal.pone.0019576

Koeck B, Pastor J, Saragoni G, Dalias N, Payrot J, Lenfant P. 2014. Diel and seasonal movement pattern of the dusky grouper *Epinephelus marginatus* inside a marine reserve. Mar Environ Res 94: 38-47. DOI: 10.1016/j.marenvres.2013.12.002

Koenig CC, Coleman FC, Kingon K. 2011. Pattern of recovery of the goliath grouper *Epinephelus itajara* population in the southeastern US. Bull Mar Sci 87: 891-911. DOI: 10.5343/bms.2010.1056

Kohno H, Ordonio-Aguilar RS, Ohno A, Taki Y. 1997. Why is grouper larval rearing difficult?: An approach from the development of the feeding apparatus in early stage larvae of the grouper, *Epinephelus coioides*. Ichthyol Res 44: 267-74. DOI: 10.1007/bf02678706

Kruskal JB. 1964. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. Psychometrika 29: 1-27.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. EGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35(6): 1547-9.

Lacoursière-Roussel A, Côté G, Leclerc V, Bernatchez L. 2016. Quantifying relative fish abundance with eDNA: A promising tool for fisheries management. J Appl Ecol 53(4): 1148-57.

Laramie MB, Pilliod DS, Goldberg CS. 2015. Characterizing the distribution of an endangered salmonid using environmental DNA analysis. Biol Conserv 183: 29-37. DOI: 10.1016/j.biocon.2014.11.025

Leray M, Alldredge AL, Yang JY, Meyer CP, Holbrook SJ, Schmitt RJ, …, Brooks AJ. 2019. Dietary partitioning promotes the coexistence of planktivorous species on coral reefs. Mol Ecol 28: 2694-710. DOI: 10.1111/mec.15090

Leray M, Haenel Q, Bourlat SJ. 2016. Chapter 14: Preparation of amplicon libraries for metabarcoding of marine eukaryotes using Illumina MiSeq: The adapter ligation method. New York (US): Humana Press.

Leray M, Yang JY, Meyer CP, Mills SC, Agudelo N, Ranwez V, …, Machida RJ. 2013. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: Application for characterizing coral reef fish gut contents. Front Zool 10: 1-14. DOI: 10.1186/1742-9994-10-34

Lucas MQ, Stat M, Smith MC, Weil E, Schizas NV. 2016. Symbiodinium (internal transcribed spacer 2) diversity in the coral host *Agaricia lamarcki* (Cnidaria: Scleractinia) between shallow and mesophotic reefs in the Northern Caribbean (20 - 70 m). Mar Ecol 37: 1079-87. DOI: 10.1111/ maec.12367

Luckhurst BE. 2010. Observation of a black grouper (*Mycteroperca bonaci*) spawning aggregation in Bermuda. Gulf Caribb Res 22: 43-9. DOI: 10.18785/gcr.2201.05

Luiz OJ, Woods RM, Madin EMP, Madin JS. 2016. Predicting IUCN extinction risk categories for the world's data deficient groupers (Teleostei: Epinephelidae). Conserv Lett 9: 342-50. DOI: 10.1111/conl.12230

Mahjoub M-S, Souissi S, Schmitt FG, Nan F, Hwang J. 2011. Anisotropy and shift of search behavior in Malabar grouper (*Epinephelus malabaricus*) larvae in response to prey availability. Hydrobiologia 666: 215-22. DOI: 10.1007/s10750-010-0549-4

Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet Journal 17(1): 10-2.

McLeod E, Szuster B, Salm R. 2009. Sasi and marine conservation in Raja Ampat, Indonesia. Coast Manag 37: 656-76. DOI: 10.1080/08920750903244143

Meyer CP. 2003. Molecular systematics of cowries (Gastropoda: Cypraeidae) and diversification patterns in the tropics. Biol J Linn Soc 79: 401-59. DOI: 10.1046/j.1095-8312.2003.00197.x

Miller MJ, Wouthuyzen S, Sugeha HY, Kuroki M, Tawa A, Watanabe S, …, Aoyama J. 2016. High biodiversity of leptocephali in Tomini Bay Indonesia in the center of the Coral Triangle. Reg Stud Mar Sci 8: 99-113. DOI: 10.1016/j.rsma.2016.09.006

Moran Z, Orth DJ, Schmitt JD, Hallerman EM, Aguilar R. 2015. Effectiveness of DNA barcoding for identifying piscine prey items in gut contents of piscivorous catfishes. Environ Biol Fishes 99: 161-7. DOI: 10.1007/s10641-015-0448-7

Munday PL, Pratchett MS, Dixson DL, Donelson JM, Endo GGK, Reynolds AD, Knuckey R. 2013. Elevated CO_2 affects the behavior of an ecologically and economically important coral reef fish. Mar Biol 160: 2137-44. DOI: 10.1007/s00227-012-2111-6

Nath RD, Bedbrook CN, Abrams MJ, Basinger T, Bois JS, Prober DA, …, Goentoro L. 2017. The jellyfish Cassiopea exhibits a sleep-like state report. Curr Biol 27: 1-7. DOI: 10.1016/j.cub.2017.08.014

Oufiero CE, Holzman RA, Young FA, Wainwright PC. 2012. New insights from serranid fishes on the