Identification of Dietary Preferences in Groupers from Raja Ampat Reefs Through Mitochondrial DNA Metabarcoding

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Abstract

Groupers (Family Serranidae) are well known as top predators in the coral reef ecosystem. This family is one of the most important ecological organisms in the food web at Raja Ampat reefs. The aim of this study, therefore, was to investigate the food composition as well as the inter and intra-specific competition of two Serranidae species, including Epinephelus malabaricus and Epinephelus areolatus in Waisai, Raja Ampat. This study is expected to fill gaps on groupers food preferences. A total sample were collected at the fish landing site was 6 fish (2 sample of Epinephelus areolatus and 4 sample of Epinephelus malabacrius). DNA Amplification was performed using the COI mithocondrial gene marker (HCOI12198 and LCOI1490 primers). Bioinformatics and data analysis using QIIME 2 and R Software. This further comprised of α and β diversities. The results showed that Serranidae diet composition comprise of 13 species. Epinephelus malabaricus species demonstrated more diet composition varieties than Epinephelus areolatus. The most abundant diet found in all fishes' gut samples was come from Scianidae family. The results indicate that there is an absence of any interspecific competition between the two species studied, due to a wide variation of diet composition. Conversely, intraspesific competitions existed amongst the Epinephelus malabaricus niches. This study highlighted DNA Metabarcoding application in trophic level and food web studies, as well as facilitating the development of information for ecological references.

Keywords: *gut content, diet partition, Serranids, trophic level, Raja Ampat, Papua*

Introduction

Serranidae groupers, subfamily Epinephelinae, are economically and ecologically important fish. These carnivorous fish live in littoral and sub-littoral zones throughout the Indo-Pacific, East Atlantic, Mediterranean, and Subtropical Zones of the Americas (Heemstra *et al*., 1993; Huerlimann *et al*., 2024). These fish occupy the highest trophic level in the food chain and, as top predators, perform the most significant function in coral reef ecosystems (Randall *et al*. 1960; Ribeiro *et al*. 2021). They are typically found in isolated habitats. Groupers, as top predator in coral reefs usually feed on crustaceans and fish (Haq, 2016).

The ecological function of large predators, such as groupers, is crucial in the preservation of the health, resilience, and biodiversity of coral reef ecosystems. Furthermore, the primary importance of predatory fish is their capacity to regulate the population dynamics of smaller fish (Syafruddin *et al*. 2024). Consequently, the investigation of trophic ecology in groupers and food webs is crucial for the identification of the ecological and trophic levels of ecosystems, which can influence the interactions and ecological processes between species (Condini, 2014). Energy recycling and trophic levels are also elucidated by food webs, which are the interactions between predators and prey in an ecological community (Allgeier *et al*. 2015; Thompson *et al*., 2015). These networks naturally exhibit a wide range

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https://ejournal.undip.ac.id/index.php/ijms
DOI: 10.14710/ik.ijms.30.4.507-518
Received : 30-05-2025
Accepted : 07-11-2025

of patterns, from intricate to the most basic interactions. Concurrently, it is crucial to conduct quantitative analyses of specific species (Pimm, 2002). Additionally, because fish require nutrients for growth and survival, the availability of food supplies in this ecosystem is crucial. Several studies have been conducted on the feeding behavior, food categories of organisms, food digestion mechanisms, and trophic relationships of fish, based on the examination of gut contents and physiological studies in the laboratory (Eya *et al*., 2011).

The dietary diversity of marine organisms, particularly fish, can be investigated by examining the contents of their digestive tracts, which permits the investigation of their trophic interactions. DNA metabarcoding is one of the numerous methodologies available for analyzing the contents of the digestive tract, and it offers certain advantages (Bohmann *et al*., 2009). Low-level and sympatric species' food consumption patterns can be identified through the use of the DNA metabarcoding approach in diet and food preference research. Moreover, this can be accomplished by comparing equivalent food categories between juvenile and adult forms (Takahashi *et al*., 2020). He *et al*. (2020) has also employed this method to ascertain the status of hybridization processes between species by employing COI markers. Additionally, Sow *et al*. (2020) reported the potential to qualitatively provide trophic networks and identify the dietary range and preferences of predatory organisms.

According to Madduppa *et al*. (2023), DNA metabarcoding has the capacity to identify degraded material in the intestine of fish. Additionally, it is recognized for its ability to generate additional information regarding the potential applications of gut contents (digestive tract). Therefore, the objective of this investigation is to assess the influence of food composition and grazing competition on groupers, as well as their correlation with trophic levels, in the Waisai coral reef in Raja Ampat, Indonesia, by employing the DNA Metabarcoding method. This investigation aims to establish an ecological benchmark for the dietary patterns and food webs found within the coral reefs of the designated study area. Recent reports have demonstrated the influence of diet partitioning on the advancement of ecosystem-based fisheries (Takahashi, 2020).

Materials and Methods

Figure 1 showed the sampling and purchase of fish. The research was conducted in Raja Ampat, Southwest Papua (00°37'42"S 130° 43'53"E). These samples were further prepared in the dive base. Prior to fish landing, the groupers at Waisai were obtained from local fishermen, using the provided fishing rods and bait food, in the form of large and small fish. In addition, the process was performed in a means to avoid the over exploitation of coral varieties. Therefore, the caught specimens were stored in a special box and placed on a special pond, before selling back to the landing. Subsequently, 6 individual adult-sized grouper samples were morphologically identified using an identification book (Allen *et al.,* 2012) and measured with a calibrated board. Therefore, the gut was crushed and most of the 2 mL sample DNA Shield placed into the tube were filled with ethanol. The DNA shield was installed to save the water needed for filtration and isolation (Pachiadaki *et al.,* 2014).

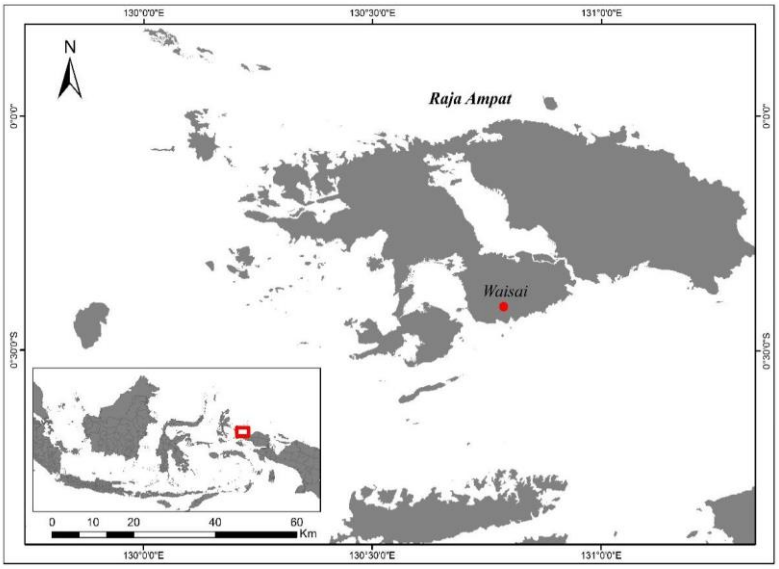

Figure 1. The Location Map of Sampling Grouper Gut Metabarcoding in Raja Ampat, Indonesia

DNA extraction

The extraction process was the first stage to obtain the biota's DNA. This was initiated by sample preparation, and then extraction using the QIAGEN kit (DNEasy Blood and Tissue kit) based on QuickStart Protocol. Moreover, this tool has the advantage of being able to extract a much larger number of DNA Metabarcoding (Hinlo, 2017).

DNA metabarcoding

The universal primer designed as HCOI12198 (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTA AAC TTC AGG GTG ACC AAA AAA-'3) and LCOI1490 (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CAA ATC ATA AAG ATA TTG G-3') were used for DNA amplification (Leray *et al.,* 2013). Furthermore, QIAGEN was used in library preparations to purify PCR amplicon and Qubit Fluorometer for the PCR product concentration evaluated (Leray *et al.,* 2013). The PCR stage for COI was Heat Lid at 110°C with start loop of 30 cycles, where each proceeded with pre-denaturated 94°C for 2 min, denaturation 98°C for 5 sec, annealing 50°C 10 sec, and extension 72°C for 5 min. PCR products performed using 313 bp COI fragment (Heindler *et al.,* 2019; Leray *et al.,* 2013). Therefore, the cleaned products were sequenced to Illumina Miseq at the University of Rhode Island.

Clustering methods

Clustering of food composition required the use of Fuzzy C Mean methods. This involves the clustering approach developed by Dunn in 1973 and improved by Bezdek in 1981. In addition, the data was allowed to belong to two or more cluster. The subsequent interpretation involves partition based on the partial membership expressed by the degree of object (Zhang *et al.,* 2016).

Data analysis

The sequencing data were analyzed using an online software to edit and align with the base chain sequence before running in a Linux system. Therefore, the paired end sequencing proceeded from Illumina Miseq, and cutadapt was used for trimming, before cleaning. Subsequently, QIIME2 was used to analyze the data of raw DNA sequence, as well as the output was observed in the form of statistic results. The characteristic features include a). Integrated and automatic tracking of original data, b). The semantic type system, c). Plug-in system to expand microbiome evaluation, and d). Support for multiple formats (ig. API). In addition, QIIME2 also encourages microbiome analysis from the first to the last DNA chain. In addition, FASTQC format was used and the sequence was trimmed using the cutadapt (command line), while MIDORI database was adopted for the taxonomic assignment. This functioned to collect information on the taxonomical classification of mitochondrial-gene sequences (Leray, 2018). Access of MIDORI (www.reference-midori.info/download.php). This version was used for each of the fifteen unique mitochondrial-encoded gene reference, estimated to contain all haplotypes per species, and theLongest MIDORI, characterized by a single haplotype per species (Wang *et al.,* 2007).

In addition, BLAST on*https://www.ncbi.nlm.nih.gov* was used for identification purposes after the sequences were grouped by taxonomy. The organisms exposed to this evaluation were recognized as pisces and grouped into species and family. Therefore, the taxonomy was subsequently interpretated in the chart to show the food composition of groupers.

Beta diversity analysis

The β diversity was analyzed using software R with Package Vegan (Oksanen, 2015; Berry *et al.,* 2017). Jaccard and Bray-Curtis dissimilarity was counted, while the β diversity was visualized using Non-Metric Multidimensional Scaling (NMDS), with METAMDS function (Harper, 2020). In addition, a dataset was created for NMDS (METAMDS) function and stress plot, while significance data we used Adonis (PERMANOVA) to establish the relationship between the food composition of *E. malabaricus* and *E. areolatus* (Yeh, 2020).

Trophic level analysis

The trophic level analysis employed the R package Bipartite (Harper *et al*., 2020). This tool functioned to show semi-quantitative trophic level prey and predator. Moreover, the development of methods used for nested tags and description of ecological network relies on bipartite package indicators (Evans *et al*., 2016). This helps to visualize the food web, as well as the relationship between prey and predators at the trophic level. In addition, network level was used with the R package (Dormann *et al*., 2008).

Result and Discussion

The total identified taxa that serve as preferred prey for *E. malabaricus* and *E. areolatus* consist of 8 families and 11 species, along with an additional group categorized as unidentified species. Figure 2 illustrates the percentage composition of various prey families found in the stomach contents of the two grouper species. The highest percentage is derived from unidentified species, with a total composition reaching 64%, and even reaching 100% in sample EB122 and around 66% in EB133. Additionally, the

most frequently detected family is Sciaenidae, appearing consistently with a total composition reaching 24%. Opleghnatidae, Blennidae, and Chanidae were recorded in even smaller proportion, each contributing less than 1% of the overall prey composition.

Among the *E. malabaricus* samples, there is a striking variation in prey diversity. EB133 shows the highest diversity, with total eight families. The highest family was Scianidae with percentage 24,66% and the lowest with total Opleghnatidae with percentage less than 1%. Conversely, EB122 shows no diversity at all, as its stomach contents consist entirely of unidentified species. The *E. areolatus* samples also display differences in dietary composition. EB127 contains a large proportion of Sciaenidae with percentage 67% along with a substantial number of unidentified species with percentage 33%.

Stomach content analysis revealed that the majority of prey found in the stomachs of *Epinephelus malabaricus* and *E. areolatus* belonged to unidentified species, accounting for 64% of the total, and reaching 100% in sample EB122 and 66% in EB133. The presence of unidentified prey is primarily attributed to the rapid digestive process, which leads to the degradation of soft tissues and the loss of key morphological traits, making visual identification difficult (Berry *et al.,* 2015; Paquin *et al.,* 2014). Furthermore, the stomach environment contains various proteolytic enzymes and digestive bacteria that accelerate the breakdown of biological material, including prey DNA, which can hinder the success of molecular techniques such as DNA barcoding or metabarcoding (Symondson, 2002). Chuaykaur *et al.* (2020) emphasized that zooplankton, small shrimp, and fish larvae constitute important dietary components for marine and brackish water groupers, particularly during the juvenile stage, and identified fish larvae as a dominant food source for *E. areolatus*. These findings suggest that many of the unidentified items could have been soft-bodied organisms such as larvae or planktonic prey that were digested beyond recognition. In general, groupers are omnivorous and display dietary flexibility, often exploiting peaks in prey availability. Additionally, the body shape and mouth gape of individual fish influence their foraging success, with larger fish and wider mouth openings being associated with the ingestion of larger prey (Paul *et al.,* 2017; Chuaykaur *et al.,* 2020). The variation in prey composition among individuals in this study may reflect differences in body size and feeding capacity. Moreover, fishing practices such as baited hook capture, commonly used by local fishers in Raja Ampat prior to fish landings, may influence the stomach contents, as bait type can introduce bias in diet studies (Kihia *et al.,* 2015). Therefore, both natural foraging behavior and capture methods must be considered when interpreting stomach content data. Overfishing also poses a serious threat to grouper biodiversity and may indirectly affect trophic dynamics by altering prey availability (Jefri *et al.,* 2015).

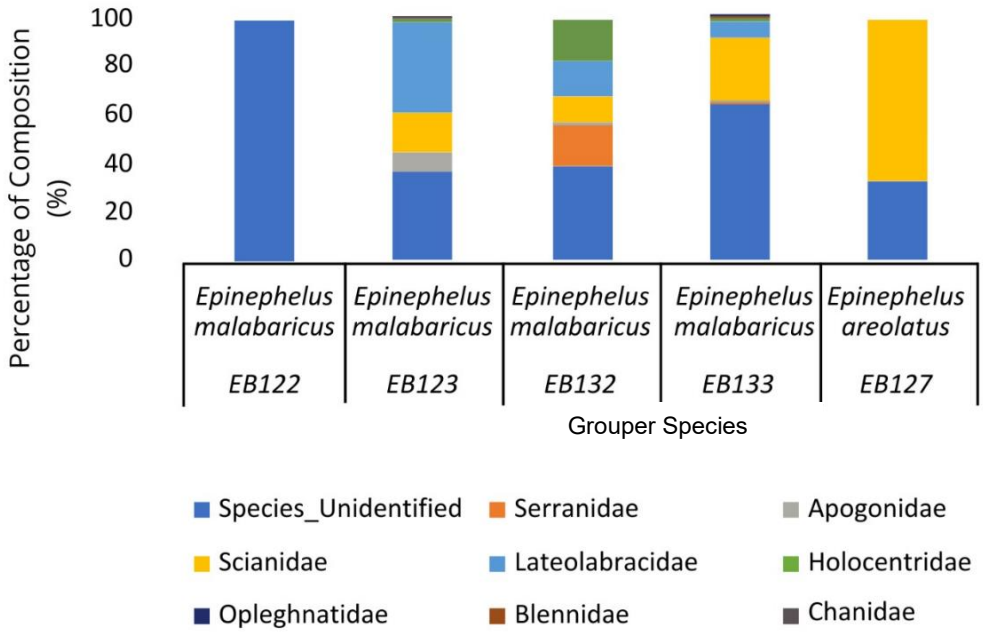

Figure 2. Percentage composition of prey families found in the stomach contents of two grouper species *E.malabaricus* and *E. areolatus*

Based on Figure 3, the stacked histogram by species illustrates the variation in diet composition across fish samples. It is evident that the majority of individuals belong to the *Species_Unidentified* category, particularly in sample EB133, which recorded the highest abundance compared to the other samples. Additionally, species from the Serranidae family such as *Epinephelus lanceolatus*, *E. malabaricus*, and *E. fuscoguttatus* were also detected in lower numbers but still contributed to the overall diet composition. Other samples such as EB122, EB123, and EB132 showed considerably lower abundances compared to EB133.

At the class level (Figure 4), the stacked histogram demonstrates that *Species_Unidentified* again dominated the composition in sample EB133. The Serranidae class was among the most prominent groups after the unidentified category, followed by Sciaenidae, Holocentridae, and Oplegnathidae. This indicates that although several families were identified, the largest proportion still came from unidentified taxa, with Serranidae making a relatively significant contribution. This is evident from the high proportion of *unidentified* prey in this study (up to 64%), likely representing morphologically unrecognizable prey such as zooplankton or larval fishes (Chuaykaur *et al.,* 2020).

Two species sample from the family Serranidae, *Epinephelus malabaricus* and *E. areolatus*, were identified as dominant predators in the coral reef ecosystem, with a notable dietary preference for fish species such as *Larimichthys crocea* and *Myripristis murdjan*. DNA metabarcoding analysis further revealed the presence of diverse prey taxa, including Mollusks, Echinoderms, and Polychaetes, consistent with previous findings by Al Kamel *et al.* (2019).

The dominance of Serranidae aligns with the ecological role of groupers as top predators in coral reef ecosystems. Their presence is crucial for maintaining reef fish community balance. Additionally, the diversity of prey items supports the notion that groupers are opportunistic feeders flexible predators capable of adjusting their diet based on prey availability. They are observed to shift prey consumption, as revealed by DNA metabarcoding method (Canals *et al.,* 2024). These dietary components were detected through genetic traces obtained from stomach content tissues, although some prey tissues may have been lost during the extraction process, potentially affecting detection efficiency (Sousa *et al*., 2016). The results indicated that *E. malabaricus* exhibited a broader prey spectrum, with genetic evidence from 10 prey families, while *E. areolatus* showed significantly lower prey diversity, with only 2 families identified through COI markers. This disparity suggests a potential interspecific competition for food resources. The application of DNA metabarcoding has proven to be a powerful tool for elucidating trophic interactions in marine ecosystems, offering higher resolution than conventional morphological methods (Bessey *et al.,* 2019). However, challenges remain in molecular diet studies, including suboptimal diagnostic sensitivity, incomplete reference databases, and the risk of

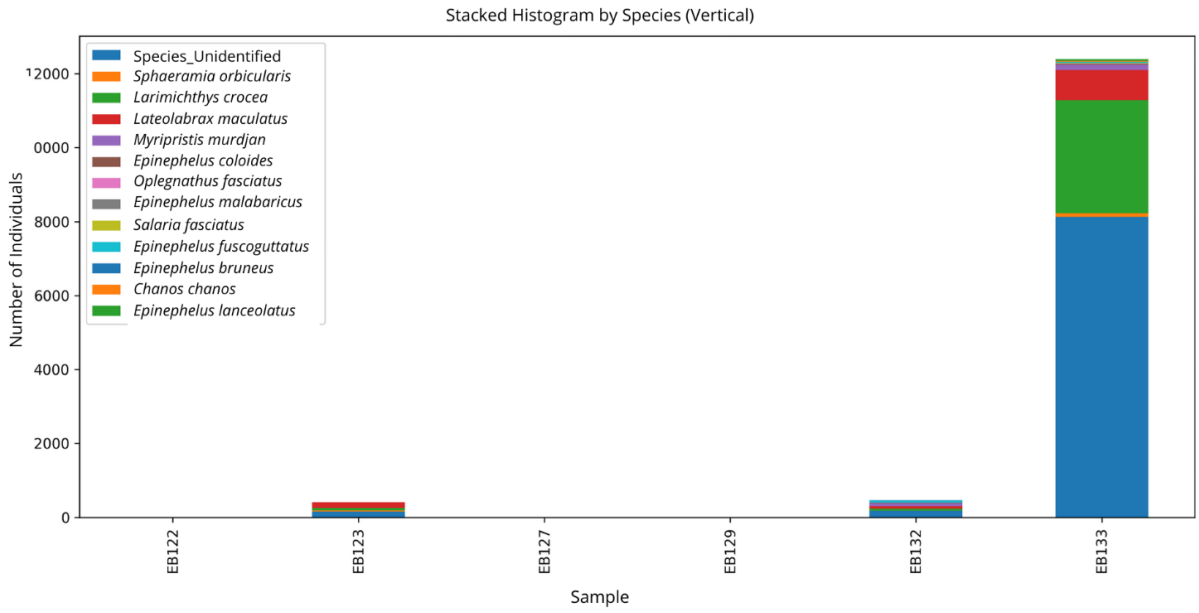

Figure 3. Food composition at species level between *E. malabaricus (EB122, EB123, EB132 and EB133) and E. areolatus* (EB127 and EB129) based on OTuS data per species

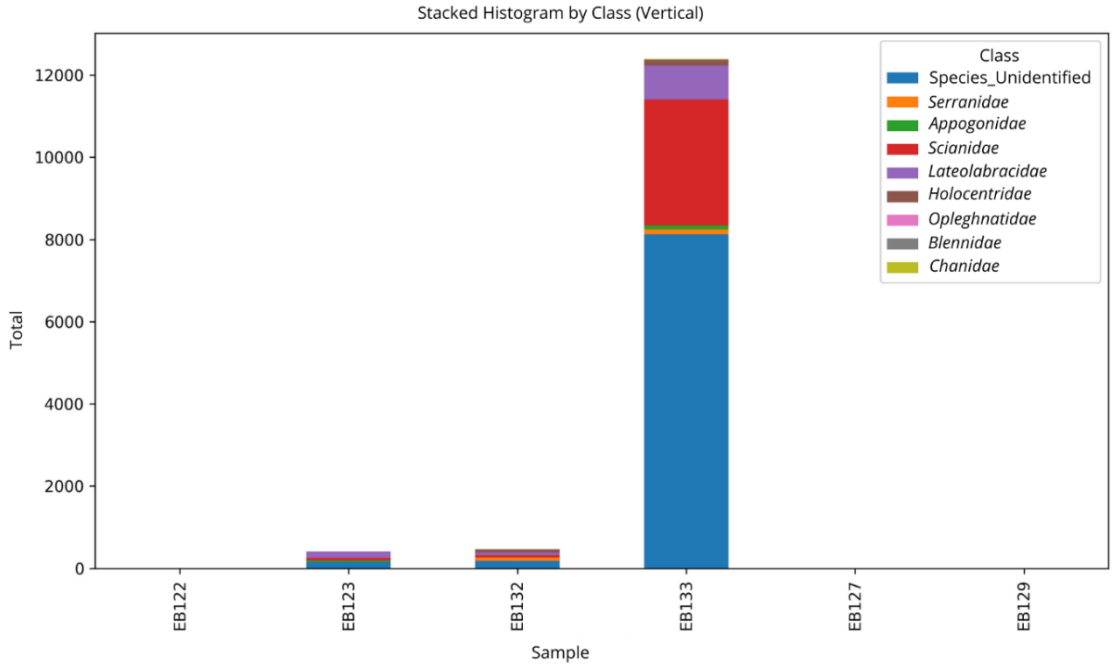

Figure 4. Food composition at species level between *E. malabaricus (EB122, EB123, EB132 and EB133)* and *E. areolatus* (EB127 and EB129) based on OTuS data per class

sample contamination, which may lead to undetected or false-negative results (Traugott *et al.,* 2021). Therefore, careful methodological design and data interpretation are essential to reconstruct food web dynamics in complex reef environments accurately.

Figure 5 presents a cluster plot illustrating two distinct dietary groupings among the grouper species, with the family Sciaenidae positioned in the second cluster. To assess dietary variation between *Epinephelus malabaricus* and *E. areolatus*, β-diversity analysis was conducted as described by Holyoak (2019), providing insight into the dissimilarity of prey composition across individuals. Furthermore, prey diversity within samples was evaluated using Shannon and Simpson indices, calculated and visualized through the ggplot function in R. The Shannon index revealed clear differences in alpha diversity, where *E. malabaricus* (EB132) exhibited the highest diversity value (1.6), while EB122 and *E. areolatus* (EB127) showed the lowest values (0.0), suggesting highly uniform or limited prey intake in these individuals. The Simpson index, which quantifies dominance patterns, showed that *E. areolatus* (EB127) had a value of 1.0—indicating complete dominance by a single prey type—while *E. malabaricus* (EB122) recorded a value of 0.0, indicating a lack of dominance. According to Morris *et al.* (2014) and Magurran (2021), Simpson index values approaching 1 indicate strong dominance by one or a few taxa. In contrast, values close to 0 reflect high evenness and more balanced prey diversity. These findings suggest that E. malabaricus exhibits more variable and diverse dietary habits compared to *E. areolatus*, potentially reflecting differences in ecological roles, feeding strategies, or habitat use within the coral reef ecosystem.

This study revealed a β-diversity pattern between *E. malabaricus* and *E. areolatus*, supported by alpha diversity values derived from Shannon and Simpson indices. *E. malabaricus* exhibited a more diverse diet, which may reflect higher intraspecific competition within its population. Meanwhile, potential interspecific competition between the two species could be driven by overlapping trophic niches or habitat use, as previously proposed by Donaldson *et al.* (1995). Such competitive interactions among reef fish are known to induce ecological stress, particularly under conditions of food scarcity and increased mortality, where predation further exacerbates individual vulnerability (Forrester, 2015).

To evaluate food preferences, this study employed modified HCOI12198 and LCOI1490 primers (Leray, 2013), which successfully identified 12 fish species across 8 families using COI-based high-throughput sequencing (~313 bp). This molecular approach has been widely validated for detecting fish and arthropod DNA in gut content studies, allowing for precise species-level resolution. Leray (2013) highlighted the effectiveness of COI primers in diverse trophic analyses, and recent findings by Clarke (2020) reported over 90% prey detection in single fish samples, confirming the utility of this method for gut content biodiversity assessments.

The dietary analysis of *E. malabaricus* and *E. areolatus* based on metabarcoding and diversity indices reveals clear interspecific differences in prey composition. As visualized in Figure 5, alpha diversity metrics showed that *E. malabaricus* exhibited higher prey diversity than *E. areolatus*. Specifically, sample EB132 (from *E. malabaricus*) recorded the highest Shannon index value (1.6), suggesting a more balanced and diverse prey intake. In contrast, both EB122 (*E. malabaricus*) and EB127 (*E. areolatus*) showed Shannon index values of 0.0, indicating very limited or highly homogeneous prey composition—likely a result of either consumption of a single dominant prey taxon or advanced digestion obscuring prey identity.

Simpson index values supported these findings. EB127 (*E. areolatus*) showed a Simpson index of 1.0, reflecting strong dominance by a single prey item. Meanwhile, EB122 showed a Simpson value of 0.0, indicating no dominance detected, possibly due to fully digested content or degraded DNA. According to Morris *et al.* (2014) and Magurran (2021), such patterns suggest differences in feeding strategies, where high Simpson values reflect dietary specialization and low values imply generalist or evenly distributed diets.

The observed variation may reflect intraspecific differences in prey availability, hunting success, or individual foraging behavior, especially among *E. malabaricus*, whose individuals showed more diverse dietary profiles. The possibility of interspecific competition also arises, especially when both species occupy overlapping ecological niches and exploit similar prey taxa. Although the data did not show explicit prey overlap at the species level, the presence of shared families and spatial proximity in sampling suggests the potential for trophic overlap, as proposed by Donaldson *et al*. (1995).

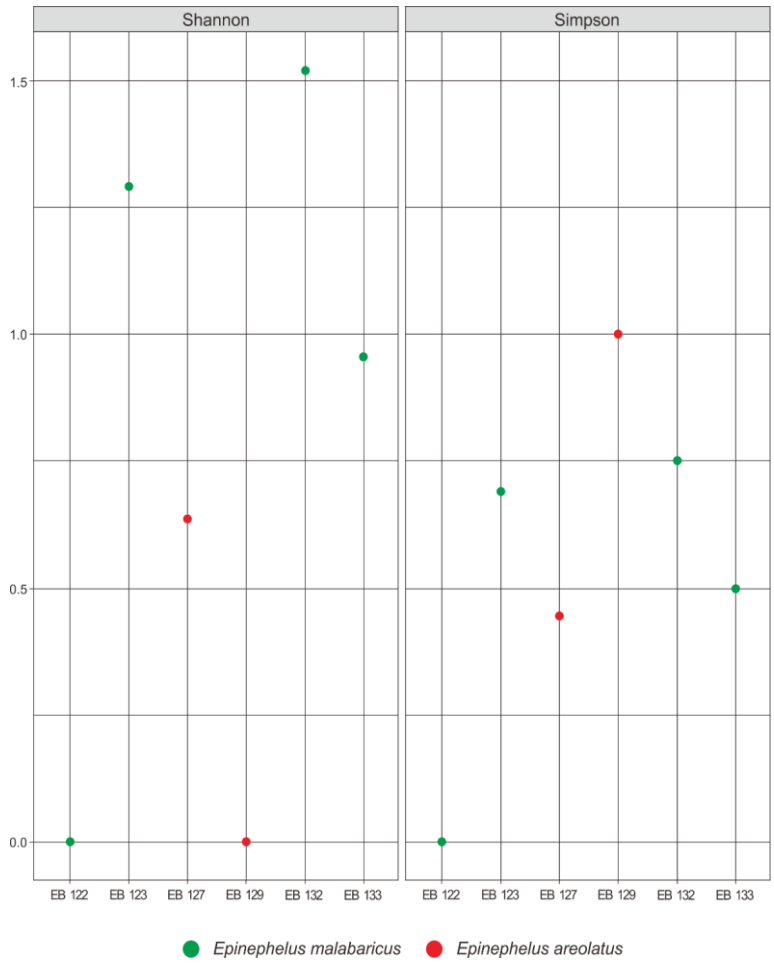

Figure 5. Alpha diversity indices (Shannon and Simpson) of stomach content composition in *E. malabaricus* (green) and *E. areolatus* (red) across individual samples (EB122–EB133). The Shannon index (left) measures prey richness and evenness, while the Simpson index (right) reflects dominance patterns. *E. malabaricus* exhibited higher overall diversity (*e.g.*, EB132 with Shannon = 1.6), whereas *E. areolatus* showed greater dominance in certain samples (*e.g.*, EB127 with Simpson = 1.0), indicating variation in prey specialization between species.

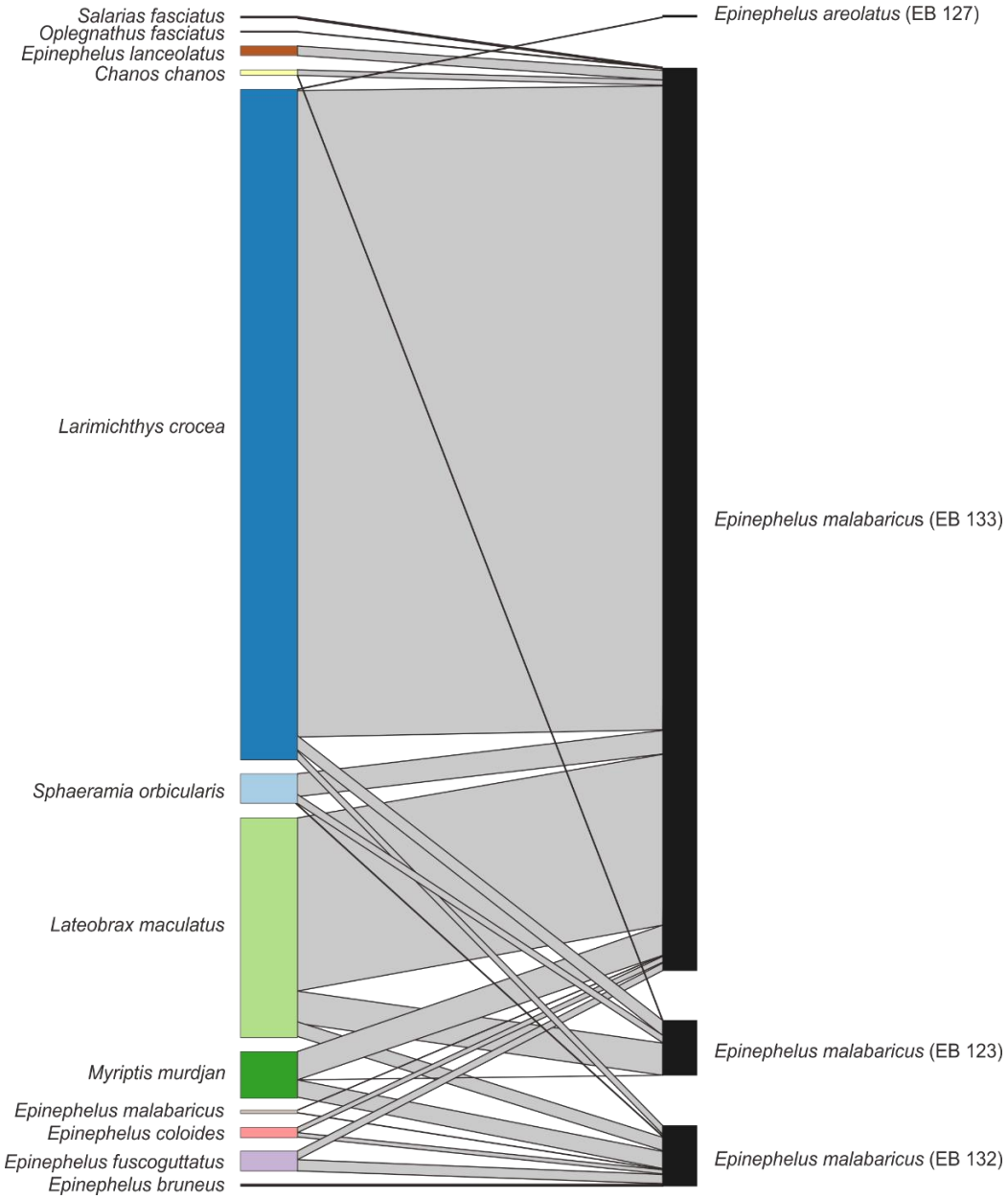

Figure 6. Sankey diagram illustrating the prey–predator relationships between identified prey species (left) and grouper stomach content samples (right) from *E. malabaricus* and *E. areolatus*. The width of each flow represents the relative detection frequency of prey taxa in individual stomach samples based on COI metabarcoding. The diagram highlights dominant prey such as *Larimichthys crocea* and *Lateolabrax maculatus*, with multiple shared prey taxa found across several *E. malabaricus* samples, suggesting broader dietary range. In contrast, *E. areolatus* (EB127) exhibited more restricted prey diversity, consistent with lower Shannon index values.

In addition to visual-based gut content observation, this study successfully employed COI metabarcoding using modified primers (HCOI12198 and LCOI1490; Leray, 2013) to identify 12 fish species across 8 families from the stomach contents. The application of high-throughput sequencing (~313 bp COI fragments) enabled precise detection of prey DNA even in cases of advanced digestion. This method's efficiency aligns with previous studies (*e.g.*, Clarke, 2020), which reported more than 90% detection rates in highly digested gut samples using similar approaches.

However, despite the strength of metabarcoding, some samples—particularly EB122—yielded no identifiable prey. This is likely due to rapid digestive enzyme activity and the presence of proteolytic microbes in the gastrointestinal tract, which degrade both morphological structures and nucleic acids (Symondson, 2002; King *et al*., 2008;

Morris *et al.,* 2014). These findings highlight a limitation in molecular diet analysis, especially under conditions of advanced digestion or sample contamination (Traugott *et al.,* 2021).

Figure 6 illustrates a bipartite trophic network consisting of 12 prey taxa and four individual predator samples. Among these*, E. malabaricus* (EB133, EB123, and EB132) was shown to have the highest prey diversity, supporting its role as a generalist feeder. In contrast, *E. areolatus* (EB127) exhibited a narrower prey spectrum, targeting primarily *Larimichthys crocea*. The observed pattern may suggest intraspecific competition within *E. malabaricus*, as several individuals shared prey types. Shared prey resources between the two species could also indicate potential interspecific competition. Similar trophic overlaps were previously documented by Speed *et al*. (2022), who reported dietary convergence between *E. areolatus* and other grouper species, possibly due to morphological similarities.

Grouper species (family Serranidae) are known carnivores inhabiting coral reefs, seagrass beds, and reef crevices (López, 2005). *E. malabaricus* is reported to feed on a wide range of prey, including macroplankton and other fishes (Haq *et al*., 2015), while *E. areolatus* consumes crustaceans, mollusks, and small fishes, sometimes including juvenile groupers (Heemstera *et al*., 1993). Interestingly, *E. malabaricus* was detected preying upon other serranids such as *E. coioides*, *E. bruneus*, *E. fuscoguttatus*, and even conspecifics. Similar behavior has been documented in other groupers, where larger individuals (*e.g*., *E. striatus*) consume smaller groupers (*e.g*., *C. fulva*) (Stalling, 2008; Hixon, 2015). These observations support the potential for cannibalism and trophic cascade effects within grouper communities.

Taken together, the observed variations in prey composition, dietary overlap, and trophic interactions between *E. malabaricus* and *E. areolatus* underscore the complex ecological dynamics shaping their feeding strategies. These insights reinforce the utility of DNA metabarcoding in resolving predator–prey relationships, particularly where conventional morphological methods fall short. However, the ecological implications of shared resources, potential cannibalism, and competition require further investigation through expanded sampling and integrative ecological modeling.

Conclusion

Groupers caught from fish landings in Raja Ampat consisted of different samples, including *E. malabaricus* and *E. areolatus*, and only 8 families or 12 species were read through the OTU dataset as the diet of the previously mentioned groupers. These were subdivided into two groups, where the first group presented the highest food composition values, and the second group consisted of the lowest. Moreover, the dominant diet observed belonged to the Scaniaedae family, with *E. malabaricus* as the most significant prey, which also occurred sequentially among species. This study identified both studied species as prey for groupers, which was corroborated by the presence of interactions with predation. In addition, there was competition between the two species and individuals, with *E. malabaricus* showing the ability to prey on both small and large groupers. This suggests the need to create a database for the analysis of abundance and food preferences. We recommend that this database be enhanced with species abundance according to fisheries biogeography data in the region. This is some reading done in general.

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