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EVALUATING THE DIVERSITY AND ABUNDANCE OF MARINE FISH IN THE MAKASSAR STRAIT, INDONESIA THROUGH ENVIRONMENTAL DNA (EDNA) ANALYSIS

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ABSTRACT

The recent adoption of environmental DNA (eDNA) represents an innovative method for assessing the presence of aquatic vertebrate species, providing a relatively straightforward approach with significant implications for conservation biology. In our investigation, we employed eDNA metabarcoding to explore the diversity of fish in the Makassar Strait. We collected eDNA from samples taken both at the surface and from the water column (15 m depth) at two specific locations within this region. The reliability of the MiFish-U primer set methodology in estimating fish diversity in the Makassar Strait was assessed. In a single survey, based on four water samples from the Makassar Strait, we successfully identified 11 marine fish taxa at the species level. These taxa belong to 8 families across 8 orders. The predominance of reef-dwelling species suggests that coral reefs play a dominant role as the primary ecosystem in this area. Among the surveyed sites, Barru Waters exhibited the highest species richness (7 species), while Pangkep Waters only revealed 4 species. Through the application of eDNA metabarcoding, this study provided a means to assess fish diversity, delivering crucial foundational information. Our findings highlight the cost-effectiveness of the eDNA metabarcoding method as a powerful scientific tool for the management and conservation of marine fish resources in the Makassar Strait.

Keywords: Environmental DNA, marine fish species, metabarcoding, Makassar Strait

INTRODUCTION

The significant decrease in global biodiversity stands out as a profound environmental crisis spanning the 20th and 21st centuries, profoundly affecting ecosystem services and the overall health of the planet (Pimm et al., 2014). Over the period from 1970 to 2010, there was an extensive reduction in biodiversity, reaching 52%, with freshwater populations experiencing an even more pronounced decline compared to marine or terrestrial ecosystems (WWF, 2014). Despite continuous efforts by scientists to comprehend and address this crisis (Monastersky, 2014), there is a pressing need to augment the effectiveness of strategies aimed at mitigating global biodiversity loss and render them more comprehensive (Beumer and Martens, 2013). While anthropogenic factors pose a threat to biodiversity (Barnosky et al., 2011), the central challenge lies in the absence of efficient and reliable tools for documenting remaining species and assessing trends in biodiversity.

The decrease in biodiversity presents a notable environmental challenge (Butchart et al., 2010), particularly impacting freshwater environments (Dudgeon et al., 2006). To effectively safeguard biodiversity, there is a necessity for rapid and noninvasive underwater biomonitoring methods (Dudgeon et al., 2006), given that traditional survey methods are costly and subject to variations in outcomes, such as the types and quantities of fish species collected, depending on the skills of investigators and the survey tools utilized. Conventional surveys of species composition, often involving direct catches, typically involve labor-intensive and time-consuming efforts, creating obstacles for promptly assessing declines in biodiversity (Minamoto et al., 2012).

Comprehending the composition of species in a particular area is a fundamental and crucial aspect of biodiversity research. Multiple factors, including habitat destruction, invasive alien species, overexploitation, climate change, and pollution—largely stemming from human activities (Clark, 2007)—contribute to declines in biodiversity. Acquiring knowledge about the species inhabiting an area is essential for biodiversity conservation, requiring the assessment of each species' presence or absence within a manageable timeframe and effort. Effectively managing aquatic biodiversity calls for reliable survey techniques to discern the

distribution of species. Survey methods with high sensitivity, indicating they achieve elevated probabilities of detecting target species when present, hold strategic value for biodiversity monitoring applications. Environmental DNA (eDNA) sampling, relying on the identification of species-specific genetic material in the environment, emerges as an exceptionally sensitive technique for biodiversity monitoring (Ficetola et al., 2008).

Environmental DNA (eDNA) involves the noninvasive retrieval of DNA from diverse environmental samples such as soil, water, and air. The genetic remnants of eukaryotic organisms may encompass free eDNA and/or DNA present within cells or organelles derived from various sources such as skin, urine, feces, mucus, or extracellular DNA resulting from cellular demise (Shokralla et al., 2012). Recent investigations suggest that tracing eDNA, as opposed to employing traditional sampling methods, can enhance the effectiveness of species detection and broaden the spatial coverage and frequency of sampling aquatic wildlife data. This has generated a growing interest in incorporating eDNA into strategies for aquatic conservation and fisheries management (Lodge et al., 2012; Bohmann et al., 2014).

Previous research has established associations between eDNA concentration and species abundance/biomass (Thomsen, Kielgast, Iversen, Wiuf, et al., 2012; Takahara, Minamoto, and Doi, 2013; Pilliod, Goldberg, Arkle, et al., 2013; Kelly et al., 2014; Klymus et al., 2015). Recent investigations on natural fish populations have further indicated that the concentration of eDNA in water samples can serve as a comparable indicator of fish abundance compared to invasive capture methods commonly utilized in fisheries management. However, the applicability of the latter method is limited to similar environmental conditions among the sampled bodies of water (Lacoursière-Roussel et al., 2016). Estimating fish abundance through eDNA analysis could significantly reduce the costs associated with data collection and prevent detrimental effects on the studied organisms (Lodge et al., 2012; Shokralla et al., 2012). From a technical standpoint, the method used for eDNA capture may also impact the ability to accurately quantify the amount of eDNA in water samples. In natural ecosystems, the eDNA released by fish is likely to originate predominantly from either mitochondria or cells, and it undergoes rapid degradation/settling after release (M.A. Barnes et al., 2014) or settling (Turner et al., 2014; Turner, Uy, and Everhart, 2015). Consequently, seasonal changes in environmental conditions are likely to influence eDNA concentration due to variations in species behavior, water stratification, temperature, and ultraviolet radiation (Zhu, 2006; Pilliod, Goldberg, Laramie, et al., 2013).

Scientific documentation has identified over 18,000 fish species utilizing the sea for reproduction and/or growth (Eschmeyer et al., 2010; Gaither et al., 2016). Approximately 20% of these species are yet to be thoroughly described, underscoring the significance of global marine fish diversity in marine ecology (Mora, Tittensor, and Myers, 2008; Costello, Wilson, and Houlding, 2012). Local diversity is equally vital for managing, conserving, and comprehending marine ecosystems ecologically. The spatial aggregation of local fish communities has pinpointed biodiversity hotspots (Morato et al., 2010; Stuart-Smith et al., 2013), while temporal accumulation has unveiled the impact of industrial fishing on both species and communities (Worm and Tittensor, 2011; Pusceddu et al., 2014). However, exploring marine fish community structures is often challenging due to limitations in taxonomic expertise and the need for extensive fieldwork. Additionally, certain marine areas, such as the deep sea, pose difficulties in observing fish communities. Consequently, ecological and conservation research frequently involves expensive surveys to investigate specific hypotheses and unveil species diversity in particular regions. Given earlier findings indicating that fishing (Myers and Worm, 2003; Genner et al., 2004) and environmental factors (Alice Valentini, Pompanon, and Taberlet, 2009) prompt rapid changes in community structure, swift and continuous investigations of marine communities are becoming increasingly imperative.

The effectiveness and essentiality of the eDNA detection method would significantly improve if it could unveil the entire fish diversity within a specified area (Matthew A. Barnes and Turner, 2016; Handley, 2015). A method to achieve this involves metabarcoding combined with massively parallel sequencing. A pioneering study successfully identified 15 fish species from seawaters using two generic and four species-specific primer sets (Thomsen, Kielgast, Iversen, Wiuf, et al., 2012). Kelly et al. (2014) similarly revealed species diversity in large mesocosms through metabarcoding, employing a single generic primer pair. The performance of eDNA metabarcoding, in comparison to alternative survey methods, including its species-specific detection capability, has been examined in previous studies. Evidently, eDNA metabarcoding detected over 50% of the species observed by alternative survey methods (e.g., 100% in Thomsen et al., 2012, 63–100% in Valentini et al., 2019, 92% in Port et al., 2016, and 72% in Shaw et al., 2016). Furthermore,

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beyond its detection capabilities, Port et al. (35) proposed that eDNA metabarcoding can unveil the fine-scale community structure.

The correlation between the presence of fish eDNA in water and the fish species inhabiting that water body is evident (Thomsen et al., 2012). The spatial and temporal relationship between a fish's presence and its DNA signal in the water may vary, offering potential for effective surveys of fish assemblages in diverse situations (Jerde, Wilson, and Dressler, 2019). The application of fish eDNA metabarcoding, which involves the use of universal primers and barcoding to identify DNA from a mixture containing multiple species, holds promise as a noninvasive and efficient tool for measuring species diversity in a manner that is both cost- and time-effective. The effectiveness of studying fish distribution and diversity is heightened through environmental DNA metabarcoding compared to traditional fish sampling methods (Fujii et al., 2019; Sard et al., 2019). Another advantage of this approach is its ability to survey a larger number of sites in a shorter timeframe than traditional methods, facilitating broader geographical coverage.

The Makassar Strait, situated in the coral triangle renowned for its rich biodiversity and the Wallacea region with a notable level of endemic species (Hakim 2017), has seen limited exploration regarding the utilization of environmental DNA (eDNA) for the identification and quantification of marine fish species. Our study aims to advance the understanding of marine fish biodiversity in the Makassar Strait. Our initial approach involved conducting eDNA metabarcoding analysis on water samples from the Makassar Strait, utilizing the MiFish-U primer set. Subsequently, we identified the marine fish species detected by the MiFish-U primer set to evaluate its effectiveness in identifying the marine fish species present in the Makassar Strait.

MATERIALS AND METHODS

Study Site

This study took place in the Makassar Strait, where we identified two representative sampling locations situated in Barru and Pangkep waters (Figure 1).

eDNA sampling

At each designated site, the eDNA metabarcoding process entailed acquiring 4 liters of water samples from a predetermined station. These samples were collected from both the surface (0–1 m depth) and a depth of 15 m at each location, and they were then cautiously stored in fresh sterile

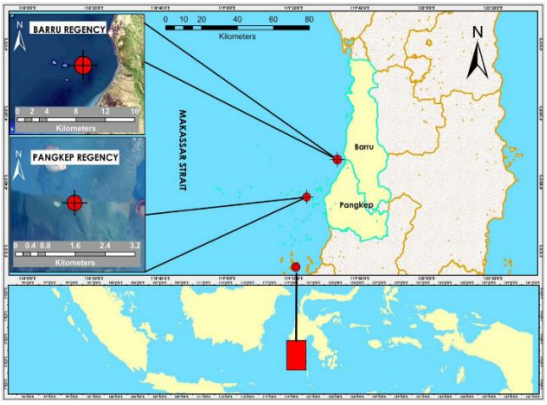

Figure 1. Study site map

polypropylene/HDPE containers, appropriately labeled. Given the delicate nature of eDNA samples, these containers were conscientiously packed in a cool box and subsequently stored in a freezer at −20 °C until the initiation of the eDNA extraction procedure.

eDNA extraction

Each 4-liters water sample was subjected to filtration using a 47 mm diameter, 0.45 µm pore size nitrocellulose membrane filter paper (Whatman™, USA). Employing a multi-filter technique, we filtered approximately 4 liters of water samples per station (4 L × 2 sites x 2 depths = approximately 16 L). The filters were regularly replaced after filtering around 2 liters of sampled water. DNA extraction from each filter followed the CTAB method, which involved adding approximately 3 mL of CTAB buffer to each filter paper. Subsequently, the filter papers were incubated in a water bath at 60°C for 3 hours, with intermittent vortexing every 30 minutes. Phase separation was achieved by introducing 1 mL of chloroform, followed by vortexing for 30 seconds and centrifugation at 12,000 rpm for 15 minutes. The resulting aqueous layer was meticulously transferred to a fresh sterile tube, and an equal volume of cold ethanol was added to precipitate the DNA. DNA pellets were formed through centrifugation at 12,000 rpm for 15 minutes. Following two washes with 70% ethanol, the DNA pellet was dissolved in sterile Molecular Biology Class water (Sigma Aldrich, USA) and stored at −20°C. The quality of DNA extracted from each station was evaluated through agarose gel electrophoresis and spectrophotometry (Thermo Scientific™ NanoDrop™ One Microvolume UV–Vis Spectrophotometer) before the preparation of DNA intended for sequencing.

Library preparation and next generation sequencing

The Qiagen Blood and Tissue DNA Extraction Kit were used to extract DNA from the samples according to the manufacturer's instructions. Library preparation involved two PCR stages. PCR products from both the initial and secondary PCR stages were subjected to purification using AMPure XP beads before proceeding to the next step. The initial PCR targeted the 12S rRNA mitochondrial DNA (mtDNA) region, a molecular marker known for its effectiveness in identifying fish and other marine vertebrates (Suarez-Bregua et al., 2022), utilizing the MiFish-U primer set (Miya et al., 2015). Each PCR reaction included 12 Kapa HotStart HiFi 2 × ReadyMix DNA polymerase, 1 µL each of 10 nM primers (F and R), 8 µL ddH2O, and 2 µL DNA Template. The PCR profile for DNA amplification consisted of: (1) pre-denaturation of the template DNA at 95°C for 5 minutes; 35 cycles of (2) denaturation at 98°C for 30 seconds, (3) annealing at 65ºC for 30 seconds, (4) primary extension at 72°C for 30 seconds; and (5) final extension (post-extension) at 72°C for 5 minutes. Contamination checks were conducted using the 96 Universal peqStAR PCR machine with negative controls (blank template). The quality of PCR products underwent assessment through electrophoresis on a 2% agarose gel (100 mL TAE buffer and 2 g agarose). Each agarose well received a 3 µL aliquot of PCR product alongside a 100 bp DNA ladder in one well. The electrophoresis machine operated at 50 Volts for 60 minutes, and the results were visualized using UV Fluorescent in an Alpha imager Mini Gel Documentation System.

PCR products that successfully passed the electrophoresis quality control underwent a second PCR for indexing purposes. In this second PCR stage, the library markers, specifically the IDT double index and Illumina sequencing adapter for Illumina - Nextera DNA Unique Dual Index, Set B, were incorporated into the target amplicons. Each reaction comprised 12.5 µl of 12 MyFi 2 × ReadyMix and 2 µl of PCR product. The PCR cycle included initial denaturation at 95°C (3 minutes), followed by 9 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. Following this, the purified indexed amplicon libraries underwent sequencing on an Illumina iSeq100.

Data analysis

To evaluate the variety of marine fish through the eDNA Operational Taxonomic Unit (OTU) dataset generated by the bioinformatics pipeline, calculations were made based on the recognized OTU taxa and their relative abundance, quantified by the number of reads per OTU.

RESULTS AND DISCUSSION

eDNA metabarcoding

From obtaining four samples at two locations in the Makassar Strait, we obtained 10,165 valid reads through MiFish-U eDNA metabarcoding, following the methodology outlined by Miya et al. (2015) (refer to Table 1). These reads facilitated the identification of 11 marine fish Operational Taxonomic Units (OTUs) categorized at the species level, exhibiting sequence identities ranging from 98.22% to 100%. These species encompassed 11 genera 8 families and 7 orders. The distribution of species in surface water (0-1m) and water column (15m depth) samples varied between sites, as illustrated in Figure 2. Figure 3 indicated that a higher number of identified marine fish species in eDNA samples from 15m depth compared to surface water (0-1m) eDNA samples.

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Table. 1. Catalog of marine fish species recognized via the eDNA metabarcoding technique, featuring the respective read counts and their proportional representation. Order Family Species Geographical Range Identity (%) Aggregate Reads Percentage of Reads

Order	Family	Species	Distribution	Identity (%)	Total Read	Read proportion (%)
Acanthuriformes	Leiognathidae	*Deveximentum indicium*	Western Pacific	100	312	3,1%
Acanthuriformes	Leiognathidae	*Nuchequula gerreoides*	Indo-West Pacific	98.82	55	0,5%
Beloniformes	Zenarchopteridae	*Zenarchopterus dispar*	Indo-Pacific	100	1751	17,2%
Clupeiformes	Dorosomatidae	*Amblygaster sirm*	Indo-West Pacific	100	280	2,8%
Clupeiformes	Dorosomatidae	*Sardinella gibbosa*	Indo-West Pacific	100	168	1,7%
Clupeiformes	Engraulidae	*Encrasicholina punctifer*	Indo-Pacific	98.22-100	1306	12,8%
Clupeiformes	Dorosomatidae	*Sardinella jussieu*	Western Indian Ocean	100	605	6,0%
Gobiiformes	Gobiidae	*Cryptocentrus melanopus*	Western Pacific	100	30	0,3%
Mugiliformes	Mugilidae	*Planiliza subviridis*	Indo-Pacific	100	81	0,8%
Syngnathiformes	Mullidae	*Upeneus sulphureus*	Indo-West Pacific	100	139	1,4%
Tetraodontiformes	Balistidae	*Pseudobalistes flavimarginatus*	Indo-Pacific	97.08-100	230	2,3%

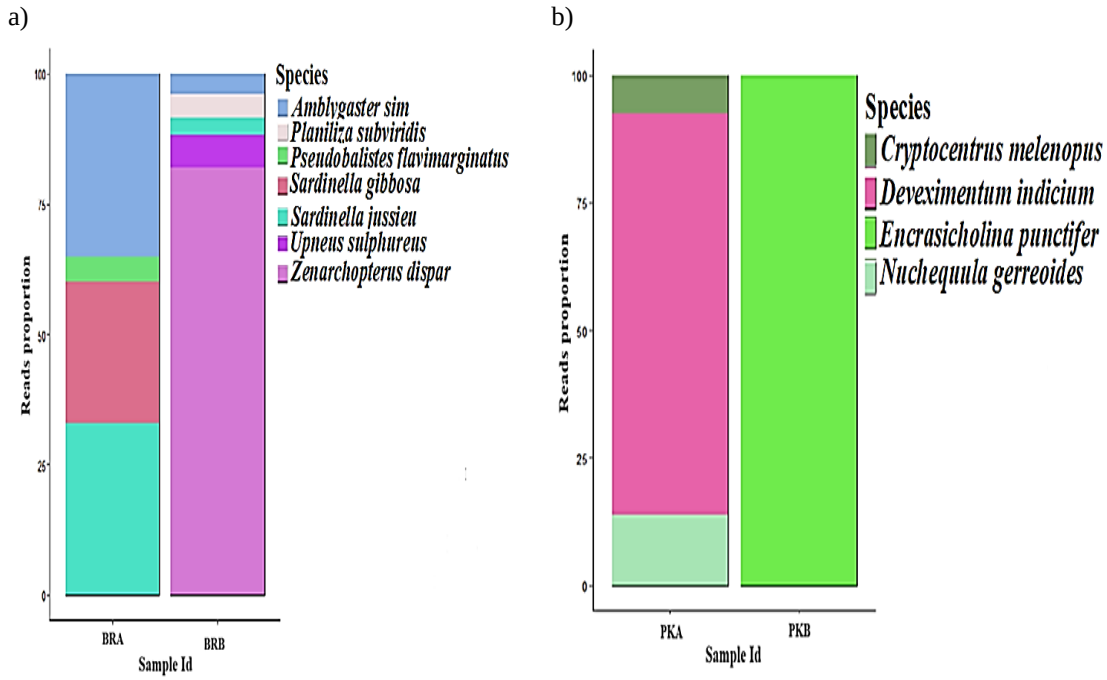

Figure 2. The percentage of marine fish species which was identified using MiFish U for eDNA metabarcoding. a) BR = Barru Waters and b) PK = Pangkep Waters

Within the family categories, Zenarcopteridae exhibited the most substantial percentage of reads, whereas Gobiidae registered the lowest, making up 0.3% (as illustrated in Figure 2 and Figure 3).

Intriguingly, Clupeiformes emerged as the predominant fish order near the surface in Barru Waters, while in Pangkep Waters, this fish order was most abundant at a depth of 15 meters (depicted in Figure 4).

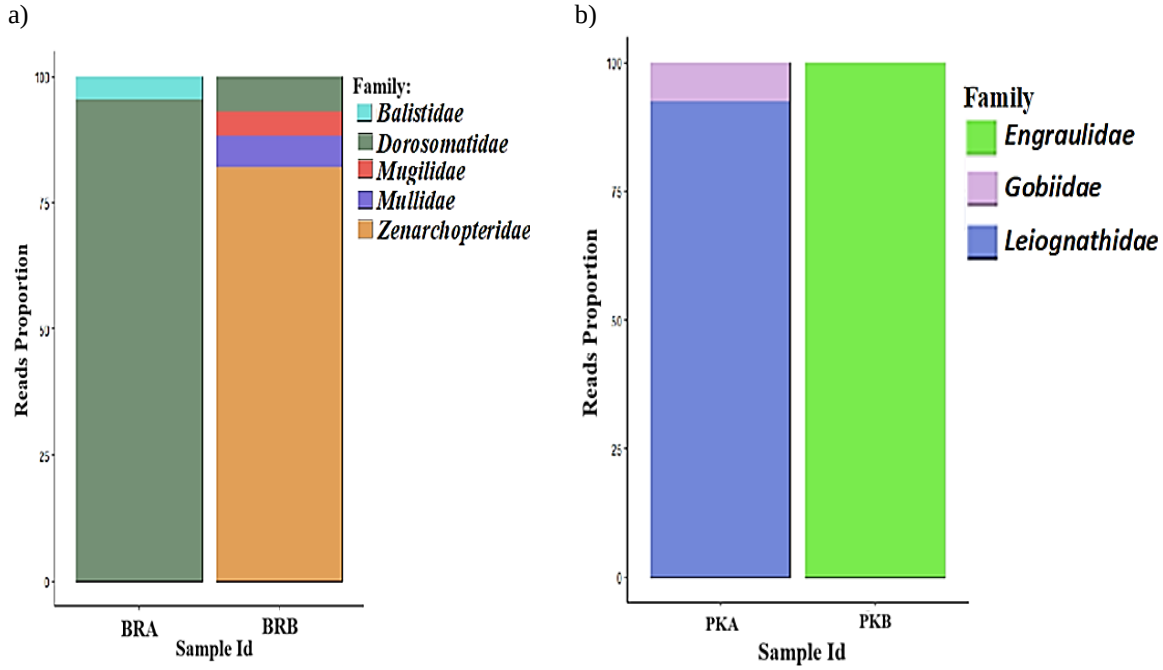

Figure 3. The percentage of marine fish family from all sites. a) BR = Barru Waters and b) PK = Pangkep Waters

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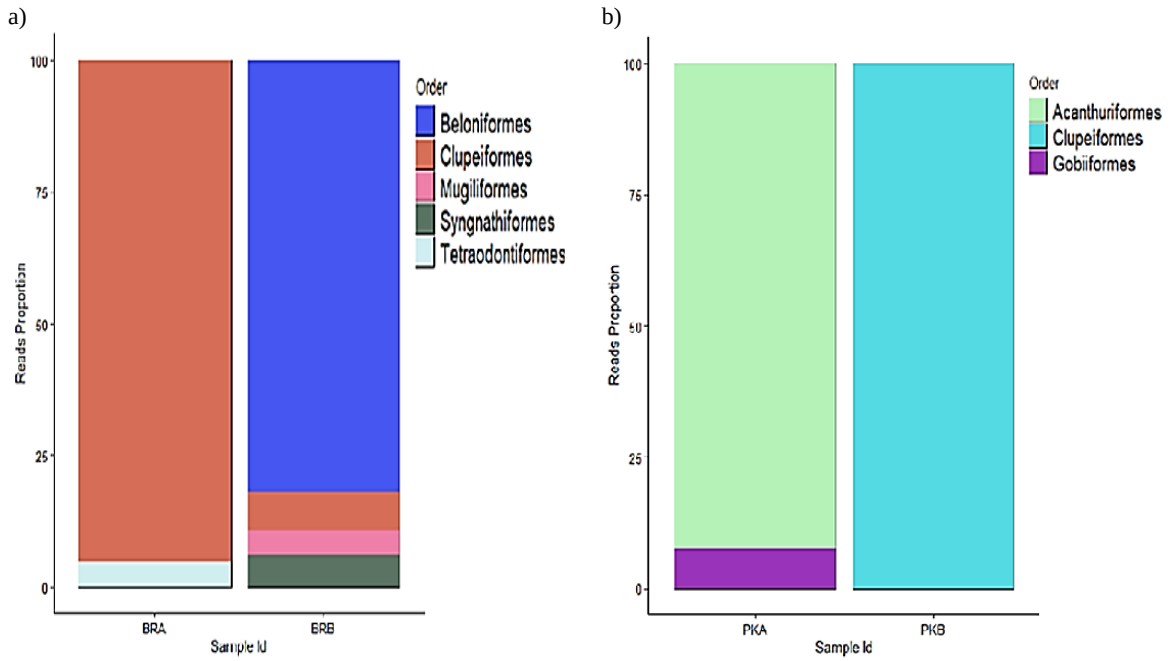
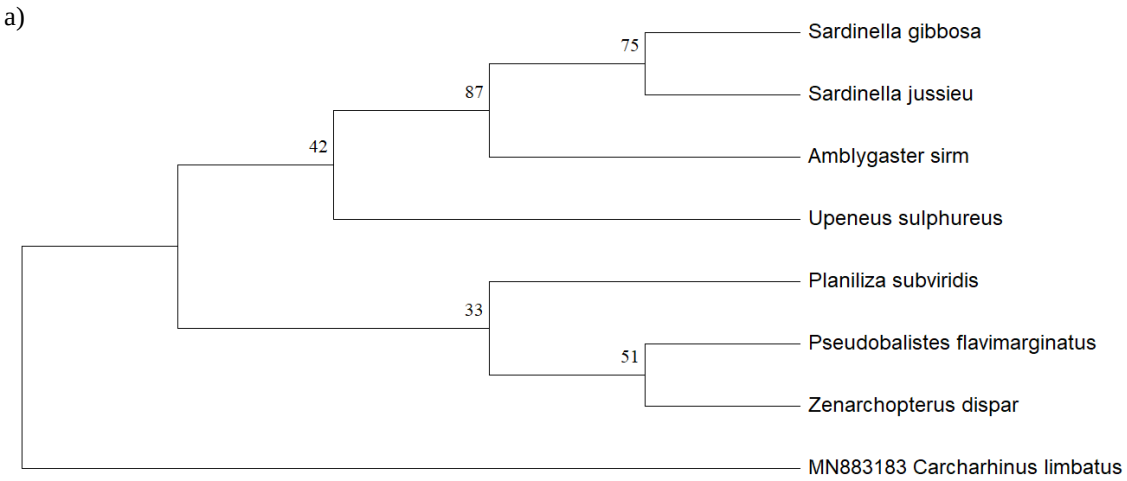


Figure 4. The percentage of marine fish order from all sites. a) BR = Barru Waters and b) PK = Pangkep Waters

Species assemblages and composition as revealed by eDNA datasets of fish



The eDNA sequences were employed to construct a phylogenetic tree delineating distinct fish families (refer to Figure 5). This tree was generated using the MFish-U primer. The outcomes of the eDNA metabarcoding identification process indicated the prevalence of fish species classified under the Perciformes order, as outlined in Table 1. Additionally, various fish groups associated with coral reefs, such as Acanthuridae and Gobiidae, were observed. Another economically valuable group within the Clupeiformes order was also detected. In this investigation, the eDNA metabarcoding results effectively discriminated five species within the Clupeidae family, specifically *Sardinella jussieu, Sardinella gibbose, Amblygaster sirm*, and *Encrasicholina punctife*r..

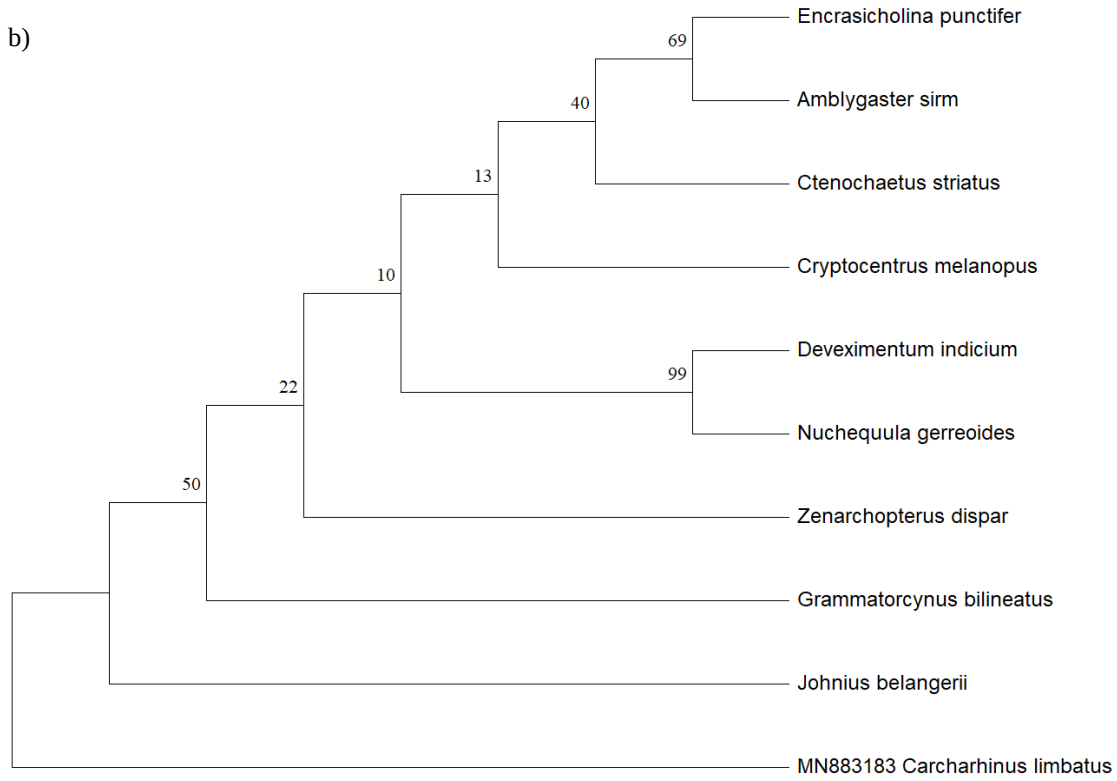


Figure 5. The Neighbor-Joining technique used in the analysis of phylogenetic trees based on sequences produced through eDNA metabarcoding.

Leveraging molecular identification is a valuable strategy for precise species recognition and is widely employed, albeit encountering challenges associated with incomplete databases (Teletchea 2009). Currently, metabarcoding stands out as a highly efficient method for assessing the species composition in a habitat, circumventing the need for resource-intensive and time-consuming surveys (Foote et al. 2012; Rees et al. 2014; Roussel et al. 2015; Piggott 2016). Nevertheless, challenges persist in its application, including concerns about susceptibility to non-target DNA contamination, biases linked to primer utilization, sequencing artifacts, potential misidentification of species, and sampling biases (Sato et al. 2017). Furthermore, this method requires adequate equipment support and the processing of bioinformatic data. The benefits of eDNA metabarcoding are magnified when combined with other approaches. A significant percentage of the identified species exhibited similarity values within the range of 95-100%, with a notable portion, specifically 72.41%, sharing 100% identity and 1.72% sharing 99% identity with GenBank voucher sequences (Andriyono, Alam, and Kim 2019).

Concerning species identification, our study revealed a comparatively limited array of fish species (11) in contrast to a previous investigation conducted by Andriyono et al. (2019), which identified 53 marine fish species (with a sequence identity of 97.08-100%). The 11 species we detected belong to 8 families, a smaller number than the 27 families documented in the study by Andriyono et al. (2019). Disparities in the counts of identified fish species and families can be ascribed to various factors, including the utilization of different genetic markers, differences in the geographical regions under investigation, and the diversity of species present at our study sites.

The relatively limited concurrence in species identification likely stems from the incomplete nature of the DNA barcoding dataset for local fish species within the GenBank online database. We propose that the eDNA technique harbors the potential to unveil a more extensive roster of documented fish species than currently feasible, considering the limitations of the existing database. This challenge can be more efficiently tackled once a more thorough DNA barcoding database for local marine organisms has been comprehensively established. Nevertheless, the DNA sequences obtained from the eDNA samples in this study are valuable, representing foundational data collected in the present timeframe. With the expectation of additional DNA barcoding sequences for local

marine species in the future, we may clarify the identities of previously uncertain or unidentified species that have been the subjects of study thus far. The fluctuation in environmental conditions across seasons is anticipated to influence eDNA concentration, given alterations in species behavior, water stratification, temperature, and exposure to ultraviolet radiation during different times of the year (Zhu 2006; Pilliod et al. 2014).

In this investigation, the mean count of identified species from water samples gathered at five locations encompassed a range of 97.08% to 100% of all the species detected through eDNA metabarcoding. Moreover, organizing and visualizing fish communities within each family category of the phylogenetic tree can assist in identifying sites that best represent the study area. Additionally, the species cluster can provide insights into the level of effort needed for the survey, as proposed in prior studies (Sato et al. 2017; Sigsgaard et al. 2020; Bessey et al. 2020).

One constraint in our current eDNA metabarcoding research involves uncertainties regarding whether certain common fish species are native or potentially invasive. Advances in eDNA techniques, such as those highlighted by Uchii, Doi, and Minamoto (2016) and Tsuji et al. (2020), have enhanced precision in detecting intraspecific genetic diversity. Consequently, these approaches hold promise for assessing both native and invasive fish populations and their impacts on local biodiversity.

CONCLUSION

The eDNA metabarcoding technique, employing the MiFish-U primer, effectively recognized marine fish species in the Sulawesi Waters region. This approach using eDNA offers improved insights into the likely fish species inhabiting the Sulawesi Waters area. Our study identified a total of 11 fish species from 7 different orders and 8 families, with the majority belonging to economically valuable fish categories. The eDNA approach efficiently gathers data on diversity and serves as a complementary method to conventional survey techniques. However, addressing discrepancies in eDNA results may require further exploration, potentially involving alternative sampling methods and considering water circulation dynamics in and out of Sulawesi Waters. Additional research, focused on seasonal variations in fish community structures through eDNA metabarcoding, could deepen our understanding of the relationship between these communities and anthropogenic factors. Moreover, by employing Next-Generation Sequencing (NGS), increasing sampling frequency, and expanding the number of sites, eDNA from the entire water body can be thoroughly analyzed to uncover patterns for each species, including their monthly, yearly, and location-specific occurrence frequencies.

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