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- RESEARCH ARTICLE -

Environmental DNA for Detection of Endangered Grouper Species (*Epinephelus spp.*)

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Abstract

Marine ecosystems nestle species or populations known to be threatened due to human overexploitation. Reliable detection and monitoring of threatened organisms is crucial for data-driven conservation actions. Furthermore, misidentification of species represents a major problem. Here, we investigate the potential of using metabarcoding of environmental DNA (eDNA) obtained directly from seawater samples to detect endangered grouper species (*Epinephelus* spp.). Cytochrome c oxidase subunit I (COI) fragment of mtDNA was used to detect groupers species in the Mediterranean Coasts. We conducted eDNA sampling at sites by underwater diving across the range of the Grouper species habitats in Northeastern Mediterranean (Antalya-Kas Region and Iskenderun Bay). eDNA was isolated from 2 liter seawater samples which were vacuum-filtered onto 0.45-mm membrane filters. Filters were then folded inwards, placed in 2 ml tubes and stored at -20 ^{o}C until DNA extraction, which took place within 24 hours. DNA was extracted from the water sample filters using the DNeasy Blood and Tissue Kit (Qiagen, USA). Manufacturer's protocols were used during all steps. PCR amplification of eDNA samples were done using selective primers of COI region of mitochondrial DNA, and next-generation DNA sequencing of PCR application was conducted. For the successfully obtained COI sequences, maximum matching rates were revealed as 80% for *Epinephelus marginatus*, 78,95% for *Epinephelus aeneus,* 73,48% for *Epinephelus costae,* 63,45% for *Epinephelus caninus,* 60,12% for *Mycteroperca rubra* and 57,12% for *Hyporthodus haifensis*. Despite the methodological challenges inherent in eDNA analysis, the results demonstrated that eDNA method may be proved to step towards a new beginning to detect and monitor endangered grouper species.

Keywords:

Environmental DNA, COI, Endangered Grouper Species, *Epinephelus spp*.

Article history:

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Introduction

The marine environment represents considerable value in terms of biodiversity and economics through fisheries and other products derived from the sea (Costanza, 1999; FAO, 2010; Nielsen, 2012). Fish are the most species-rich group of vertebrates and comprise a keystone in monitoring of environmental health of marine ecosystems (Thomsen et al. 2012). Information on distribution of a species is a vital component of understanding their ecology and extinction risks and essential for conservation of fragile populations (Begon et al. 2005).

Recently, environmental DNA (eDNA) has been used to document the distributions of aquatic vertebrate species (Valentini et al. 2009). Detection of short, species-specific DNA fragments in the water may increase the accuracy and decrease the cost of surveys and allow detection of rare, endangered or invasive species. eDNA is genetic material found in bulk environmental samples (e.g., soil, water, air) without isolating the individual organisms or their parts. It is defined by the process used to collect it. eDNA is used in the context of monitoring aquatic and semi-aquatic populations by extraction of DNA from water bodies. The total eDNA includes DNA that originates from the saliva, urine and skin cells of animals occupying water bodies and similarly from animals that visit the environment, such as birds and mammals visiting the water body to drink (Willerslev et al. 2003).

Though of big potential for modern biodiversity monitoring, environmental DNA detection in natural populations has until recently only been applied to a few common freshwater invasive species of amphibians and fish (Ficetola et al. 2008; Goldberg et al. 2011; Jerde et al. 2011; Brabon et al. 2015; Mächler et al. 2015; Robson et al. 2016; Davison et al. 2016; Keskin et al. 2016). The analysis of water for species-specific eDNA is a new and emerging technique that will have applications for aquatic organism surveys and conservation applications (Rees et al. 2014).

Grouper species belong to the Serranidae Family in Turkish marine waters are represented with 6 species (*Epinephelus aeneus, Epinephelus marginatus, Epinephelus caninus*, *Epinephelus costae, Hyporthodus haifensis* and *Mycteroperca rubra) (Figure 1)*. The grouper species have increasingly become considered vulnerable to endangered in conservation status due to increasing exploitation by fishermen and the changing marine environment. To investigate the extent of these threats, it is important to be sure existence of these species found in this part of the Mediterranean.

Up to date, in Turkey, there has been no any study using environmental DNA for the monitoring and conservation of marine species. Therefore, this study is the first environmental DNA experiment in Turkish marine waters to investigate environmental DNA (eDNA) obtained directly from seawater samples to detect endangered grouper species from Antalya-Kas Region and Iskenderun Bay in the northeastern Mediterranean Sea.

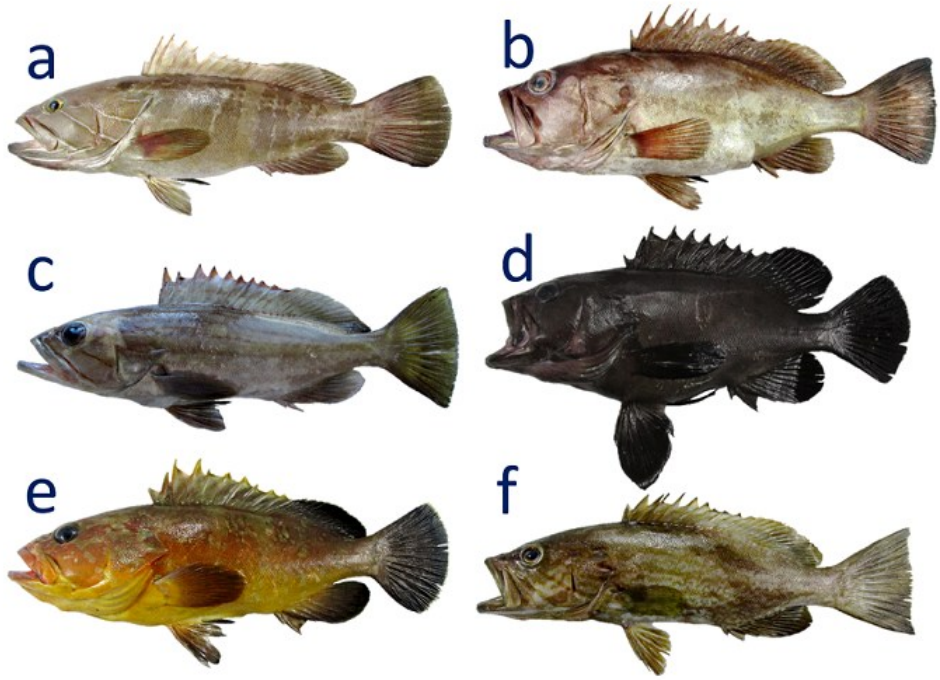

Figure 1. Grouper species in the Turkish marine water: a) *Epinephelus aeneus*, b) *Epinephelus caninus*, c) *Epinephelus costae*, d) *Hyporthodus haifensis*, e) *Epinephelus marginatus*, f) *Myscteroperca rubra*. All the pictures are taken during this study.

Material and Methods

We conducted eDNA 50 water sampling at sites by underwater diving across the range of the grouper species habitats in northeastern Mediterranean (Figure 2.). eDNA was isolated from 2 liter seawater samples which were vacuum-filtered onto 0.45-mm membrane filters. Filters were then folded inwards, placed in 2 ml tubes and stored at -20 ºC until DNA extraction, which took place within 24 hours. DNA was extracted from the water sample filters using the DNeasy Blood and Tissue Kit (Qiagen, USA). Manufacturer's protocols were used during all steps.

Figure 2. Sampling sites (●) of marine waters.

Polymerase chain reaction (PCR) amplification was performed with following selective primers especially designed for this experiment:

COI-Forward 5'-TCAACCAACCACAAAGACATTGGCAC-3'
COI-Reserved 5'- ACTTCAGGGTGACCGAAGAATCAGAA-3'

The PCRs were conducted in a 50 ml total volume with 0,4 uM of each primer, 0,2 mM of dNTP and 1.25U of *Taq* DNA polymerase in a PCR buffer that included 20mM of Tris–HCl (pH 8.0), 1,5mM of $MgCl_2$, 15 mM of KCl and 1-2 µl template DNA. Denaturation step at 94°C for 30 s, 50 °C for 30 s, and 72 °C for 45 s for 30 cycles and followed by a final extension for 7 min at 72 °C. PCR products were visualized using electrophoresis on 1,5 % agarose gel. The DNA sequencing was attempted to determine the order of the nucleotides of mtDNA COI region. The chain termination method by Sanger et al. (1977) was applied with Bigdye Cycle Sequencing Kit V3.1 and ABI 3130 XL genetic analyzer. The initial alignments of partial COI sequences were performed with Clustal W program (Thompson et al. 1994) and final alignment was completed manually with BioEdit (Hall, 1999). The BLAST search using NCBI nucleotide database was used for sequence matching.

Results

A total of 50 sea water samples were collected from the two regions, and good quality DNA were extracted from only 11 of the total samples. Good quality PCR products were obtained from all the 11 eDNA samples using selective COI primers. Thus, six samples of the Iskenderun Bay and five samples of the Antalya-Kas of mtDNA COI region were successfully sequenced.

The sequences of the 11 samples were determined as groupers COI gene sequence with different matching ratio as a result of BLAST search using NCBI nucleotide database. For all obtained COI sequences of Iskenderun Bay samples, maximum matching rates were ranged from 49 % for *Hyporthodus haifensis* to 80% for *Epinephelus marginatus* (Figure 3). *Epinephelus marginatus* matched with the highest percentage and *Hyporthodus haifensis* matched with lowest percentage in all the 6 samples. In the Kas samples, maximum matching rates were ranged from 50 % for *Hyporthodus haifensis* to 79 % for *Epinephelus marginatus* (Figure 4). *Epinephelus marginatus* matched with the highest percentage and *Hyporthodus haifensis* matched with lowest percentage in all the 6 samples.

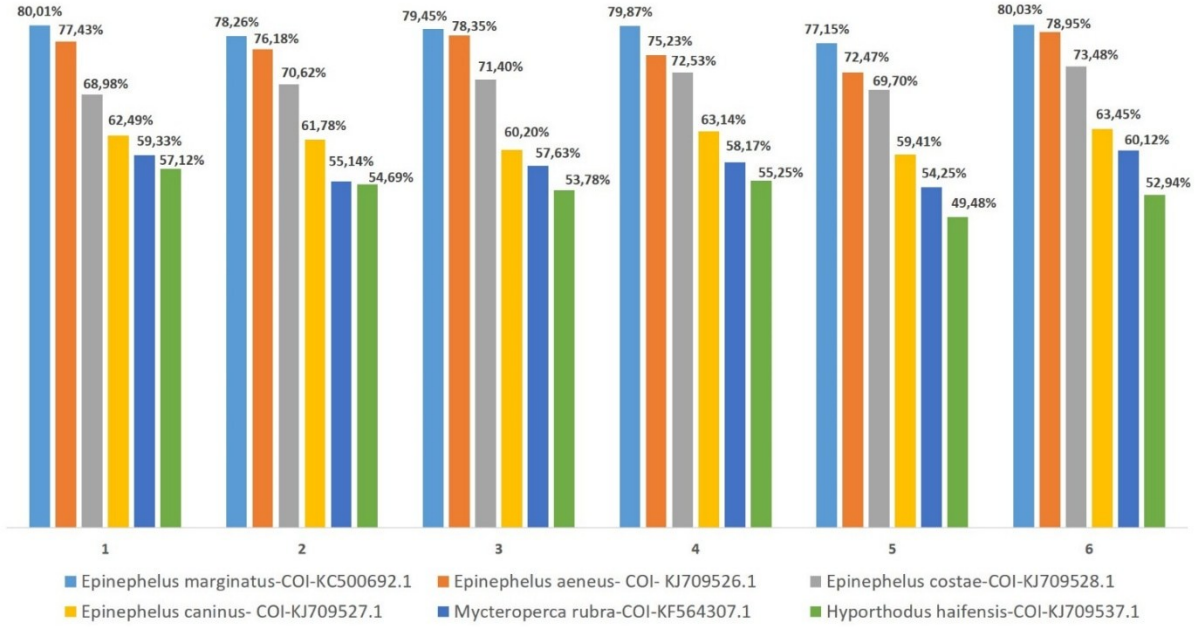

Figure 3. Matching rate of sequences for the Iskenderun Bay samples.

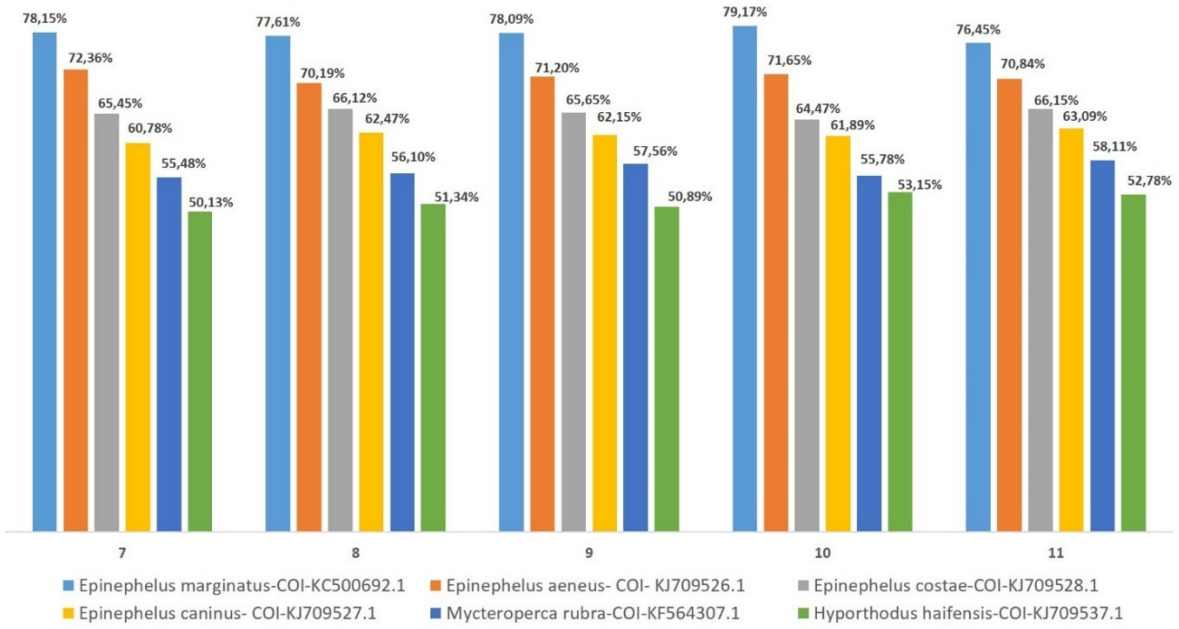

Figure 4. Matching rate of sequences for the Antalya-Kas region samples.

Discussion

In the present study, the results first time demonstrated that eDNA method may be proved to step towards a new beginning to detect and monitor endangered grouper species. Environmental DNA is a useful tool for monitoring endangered grouper species especially for Dusky grouper *E. marginatus*. The results from this study indicate that eDNA detection methods are an effective way to determine the existence and distribution of grouper species.

In the other side Iskenderun and Kas region, Dusky grouper *E. marginatus* relatively revealed higher matching rates that may due to more stable behavior of *E. marginatus*. Since *E. marginatus* do not run away when we get close to it and stay close to us when we get underwater samples from the sampling sites.

Thomsen et. al. (2012) isolated eDNA from ½-litre seawater samples collected in a temperate marine ecosystem in Denmark useing next-generation DNA sequencing of PCR amplicons of the mitochondrial gene Cytochrome b and obtained eDNA from 15 different fish species. Yamamoto et. al. (2016) tested the relationships between the distribution of fish and eDNA, they conducted a grid survey in Maizuru Bay, Sea of Japan, and sampled surface and bottom waters while monitoring biomass of the Japanese jack mackerel (*Trachurus japonicus*) using echo sounder technology. They concluded that eDNA generally provides a 'snapshot' of fish distribution and biomass in a large area. In another study, Port et. Al. (2016) reported of a 2.5 km eDNA transect surveying the vertebrate fauna present along a gradation of diverse marine habitats associated with a kelp forest ecosystem. Using PCR primers that target the mitochondrial 12S rRNA gene of marine fishes and mammals, they generated eDNA sequence data. They founded spatial concordance between individual species' eDNA and visual survey trends that eDNA was able to distinguish vertebrate community assemblages from habitats separated by as little as ~60 m.

The present techniques for isolating and identifying eDNA are an effective and efficient way to detect species presence in the marine water. We succeeded in establishing a method for estimating species composition using eDNA with minimal disturbance of natural environments. Despite of the methodological challenges inherent in eDNA analysis, the results revealed that using eDNA samples in marine environment is promising tool to detect and monitor endangered grouper species.

Acknowledgments

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