

MOLECULAR IDENTIFICATION OF GROUPE FISH (PERCIFORMES: SERRANIDAE) LANDED FROM PANGPANG BAY, BANYUWANGI

IDENTIFIKASI MOLEKULER IKAN KERAPU (PERCIFORMES: SERRANIDAE) YANG DARATKAN DI TELUK PANGPANG, BANYUWANGI

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ABSTRAK

Teluk Pangpang Banyuwangi memiliki potensi perikanan yang tinggi, perikanan pengolahan dan perikanan tangkap, khususnya ordo Perciformes dari famili Carangidae, Lutjanidae dan Serranidae. Kerapu (Serranidae) merupakan salah satu komoditas ikan laut yang bernilai ekonomi tinggi. Namun data penangkapan ikan kerapu di daerah ini hanya dikenal sebagai kerapu tanpa mengetahui jenis spesiesnya. Jenis ikan kerapu sulit untuk diidentifikasi karena ikan kerapu memiliki tingkat kemiripan morfologi dan variasi warna yang tinggi. Oleh karena itu, diperlukan identifikasi molekuler untuk menentukan jenis kerapu di wilayah ini. Penelitian ini bertujuan untuk mengidentifikasi jenis ikan kerapu yang didaratkan dari Teluk Pangpang berdasarkan barcode DNA. Selain itu, dilakukan analisis pohon filogenetik antar spesies kerapu (Serranidae), dan analisis jarak genetik spesies yang teridentifikasi dibandingkan dengan database NCBI. Pengamatan morfologi pada ikan kerapu dilakukan terlebih dahulu, dan sampel daging ikan diambil untuk percobaan identifikasi molekuler. Hasil identifikasi berbasis molekuler adalah *Epinephelus coioides* dan *Cephalopholis miniata*. Hasil rekonstruksi pohon filogenetik kedua kerapu membentuk klad yang terpisah. Jarak genetik antar spesies *Epinephelus coioides* berkisar antara 0,00-0,122, sedangkan spesies *Cephalopholis miniata* memiliki jarak genetik 0,00-0,002.

Kata kunci : ikan karang; keanekaragaman; genetic; sumberdaya

ABSTRACT

The Pangpang Bay Banyuwangi has a high potential for fisheries, processing and capturing fisheries, especially the order Perciformes of the families Carangidae, Lutjanidae and Serranidae. Grouper (Serranidae) is one of the high-economic marine fish commodities. However, the grouper fishing data in this area is only known as grouper without knowing the type of species. It is challenging to identify grouper species because groupers have a high degree of morphological similarity and colour variation. Therefore, molecular identification is necessary to determine the type of grouper in this region. This study aims to identify the grouper species that landed from Pangpang Bay based on DNA barcoding. In addition, phylogenetic tree analysis between grouper species (Serranidae) was carried out, and genetic distance analysis of the identified species was compared to the NCBI database. Morphological observations on groupers were carried out first, and fish meat samples were taken for experiments on molecular identification. The results of molecular-based identification revealed two grouper species *Epinephelus coioides* and *Cephalopholis miniata*. The results of phylogenetic tree reconstruction of the two groupers form a separate clade. The genetic distance between *Epinephelus coioides* ranged from 0.00-0.122, while the species of *Cephalopholis miniata* had a genetic distance of 0.00-0.002.

Keywords: coral fish; diversity; genetic; resources

INTRODUCTION

Pangpang Bay Banyuwangi is one of the coastal areas that is the center of marine fisheries activities in Banyuwangi. This bay has a high potential for aquaculture, fisheries processing, and capture fisheries, especially the order Perciformes of the families Carangidae, Lutjanidae, and Serranidae (Andriyono et al. 2020). Grouper (Serranidae) is one of the high economic marine fish commodities. Based on data from the Banyuwangi BPS (2019), the total production of grouper capture fisheries in 2018 reached 401 tons. The high level of exploitation of this fish can threaten its sustainability. For example, according to the International Union for the Conservation of Nature (IUCN), The humpback grouper (*Cromileptes altivelis*) has been included in the endangered category (Morris et al. 2000). Utilization and management efforts can be made to maintain grouper genetic resources. The initial step that can be taken is to identify the type of grouper caught by fishermen because the data on grouper fishing in Indonesia is only known as grouper without knowing the type of species. It is difficult to identify grouper species because groupers have a high degree of morphological similarity and color variation. Morphological identification is widely used to identify fish species, but this identification is less accurate to prove the types of fish species that have a high level of morphological similarity such as groupers (Kamal et al. 2019). Therefore, molecular identification is necessary to determine the type of grouper in this region.

MATERIALS AND METHODS

Sampling of the grouper

Grouper fish samples that have been obtained from the catches of fishermen in Pangpang Bay in Banyuwangi, are then morphologically identified by classifying each species based on morphological characteristics such as body shape and color of the fish. Grouper samples that have been identified morphologically are then taken about 1 cm of the body part in the caudal fin for DNA extraction and stored in a tube that already contains 96% ethanol for molecular identification (Andriyono and Suciyo 2020).

DNA extraction

DNA extraction was carried out using the NEXprep™ Cell/Tissue DNA Mini Kit. The initial step in the extraction is that the sample in the

microtube is taken weighing 20 mg and then inserted into a new microtube and added with Buffer GT1 as much as 200 L and homogenized. The second stage or lysis stage is adding 200 L of Buffer GT2 and 20 L of proteinase-K enzyme to the sample from the first stage, then vortexed and incubated at 56°C for 10 minutes. The third stage or binding stage is the step by adding 200 L of 100% ethanol to the sample from the second stage and then vortexing it and then centrifuging it for 2 minutes at a speed of 13,000 rpm. After the centrifugation process, take the supernatant and transfer it to a collection tube that has been installed with a spin column. The fourth stage or washing stage is adding 500 L Buffer W1 into the spin column and centrifuging for 1 minute at a speed of 13,000 rpm then discarding the supernatant in the collection tube. After that, add 500 L Buffer W2 to the spin column and then centrifuge for 1 minute at 13,000 rpm then the supernatant in the collection tube is discarded again and centrifuged again for 2 minutes at 13,000 rpm. The last stage or elution is adding 100 L of Elution Buffer to the spin column which has been transferred to a new 1.5 mL tube. Then incubated at room temperature for 5 minutes then the sample was centrifuged for 1 minute at a speed of 13,000 rpm. After being centrifuged, discard the spin column and the genomic DNA was obtained. The genomic DNA obtained was stored in a freezer at 9°C to be used as a PCR template DNA.

DNA amplification using PCR

DNA amplification uses specific primers aimed at amplifying the target. The primary target in this study was the Cytochrome Oxidase Subunit I (COI) gene in the mitochondrial genome using two universal primers, namely LCO (5' GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO (5' TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al. 1994). DNA amplification is carried out through several stages, namely denaturation, annealing and extension which are carried out as many cycles as needed. This PCR process uses a thermocycler with a predenaturation stage at a temperature of 94°C for 3 minutes, then denaturation at a temperature of 94°C for 30 seconds is repeated for 40 cycles. Furthermore, annealing at 55°C for 30 seconds was repeated for 30 cycles, extension at 72°C for 45 seconds was repeated for 30 cycles and post extension at 72°C for 5 minutes (Andriyono et al., 2020).

Visualization of PCR Products with Electrophoresis

The visualization of this PCR product was carried out with electrophoresis. Electrophoresis aims to separate chemical compounds based on the movement of molecules in an electric current (Madduppa et al. 2016). The initial stage of electrophoresis is to make 1.5% agarose gel by mixing 1.5 g of agarose powder with 100 mL of TAE buffer solution (Tris Acetate EDTA) as an electrophoresis medium gel. How to make it by mixing the two ingredients and then heating it in the microwave to mix it with the TAE buffer. The cooked agar solution is poured into the mold and put the comb on the top of the mold before placing the agar in the mold. Then, wait for 15-25 minutes until the gel is formed (Zein and Prawiradilaga 2013). After that, enter the PCR product that has been mixed with loading dye (1 μ) and then insert it in the agarose well. Electrophoresis was carried out at 110 volts for 30 minutes. The electrophoresis results were seen using a transilluminator UV light. The results of a good visualization of PCR products will show a clear band with a product size of 500-700bp (base pairs) and the resulting PCR product is ready for sequencing (Zein and Prawiradilaga 2013). The PCR products to be sequenced are sent to PT. Genetics Science to obtain sequence data.

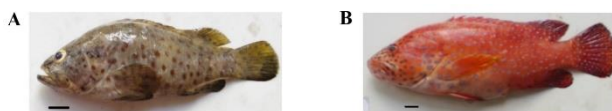


Figure 1. Morphology of grouper obtained from Pangpang Bay Banyuwangi, (a) BWKR1, (b) BWKR2. Bar scale: 2 cm.

Data Analysis

Sequence data that has been obtained is then processed using Chromas 2.6.6 (<http://technelysium.com.au/wp/chromas/>) for trimming nucleotide bases that have poor peak quality because poor peaks can cause the level of accuracy to be low and not representative to be used as data. After cutting, DNA sequence alignment was performed using MEGA-X (Molecular Evolutionary Genetics Analysis) (Kumar et al. 2018) software to determine species from their genetic code. The sequence data were edited with alignment tools in MEGA X software to see the difference between one sequence and another. Then, the data was edited using Clustal-W to see the diversity of

nucleotide bases (Tamura et al. 2013). The data from the alignment results are then matched with the data contained in Genbank at NCBI (National Center For Biotechnology Information) using BLASTN (Basic Local Alignment Search Tool Nucleotide) based on the level of similarity (<https://www.ncbi.nlm.nih.gov/>). The Genbank site at NCBI is a database facility that contains DNA sequence information from all species in the world. The BLASTN results obtained are used to see the species found based on the sample data. BLASTN results will show the percentage similarity of the nucleotide sequences and result in the determination of the nucleotide sequence in the species name. Then calculate the distance of genetic variation between species using the Kimura 2-parameter model.

Results and discussion

Morphology identification

The result of the morphology identification showed that the sample code for grouper BWKR1 has similarities with the orange spotted grouper (*Epinephelus coioides*). The body shape and color of this fish have a fairly close resemblance. The particularity of the species *Epinephelus coioides* is the presence of orange spots on the head, body, and fins. Orange spots on the body tend to be straight parallel. The sample code BWKR2 has similarities to the red grouper (*Cephalopholis miniata*) which commonly known as red coral hind/red coral cod. The body shape and color of these fish have a fairly close resemblance. The distinctive feature of this species is its reddish-orange body with blue spots all over the body and fins, the tail is round and the pectoral fins are orange. A distinctive feature of *Epinephelus coioides* is the presence of orange spots on the head, body, and fin species (Figure 1).

The results obtained are supported by morphometric and meristic measurement data (Table 1). The results of morphometric measurements of the sample code BWKR1 have a total length of 21.5 cm, standard length 17 cm, head length 6 cm, body height 5.6 cm, head height 5 cm, tail base height 2.5 cm. The results of meristic characteristics of the sample code BWKR1 have 11 spines and 14 soft rays on the dorsal fin; 18 soft rays on caudal fin; 3 spines and 7 soft rays on the anal fin; 1 spine and 6 soft rays on the ventral fin and 15 rays on the soft fin on the pectoral fin. *Epinephelus coioides* has 11 spines and 13-16 soft rays on the dorsal fin; 3 spines and 8 soft

rays on anal fin; 18-20 soft rays on pectoral fin. Based on the morphological data, it is suspected that BWKR1 is a orange spotted grouper (*Epinephelus coioides*) (Horiike et al. 2009). The results of morphometric measurements of BWKR2 sample code have a total length of 22.8 cm, standard length 18 cm, head length 6.8 cm, body height 6 cm, head height 4 cm, tail base height 2 cm. The results of meristic characteristics of the sample code BWKR2 have 9 spines and 13 soft rays on the dorsal fin; 14 soft rays on caudal fin; 3 spines and 8 soft fins on the anal fin; 1 hard and 7 soft rays on the ventral fin and 17 soft rays on the pectoral fin. *Cephalopholis miniata* has 9 spines and 14 soft rays on the dorsal fin; 3 spines and 9 soft rays on the anal fin. Based on the morphological data, it is suspected that BWKR2 is a red grouper (*Cephalopholis miniata*) (Baldauf 2003).

Molecular identification

Based on the molecular analysis data obtained the same results with the results of the morphology identification. Sample BWKR1 showed 100% similarity to the orange spotted grouper species (*Epinephelus coioides*) KY371466, while sample BWKR2 showed 100% similarity to the red grouper species (*Cephalopholis miniata*) MN870587 (Table 2). In the phylogenetic tree, the grouper *Epinephelus* and *Cephalopholis* form separate clades (Figure 2). Clade I consists of sequences of *Epinephelus coioides* obtained and added to sequences from Genbank consisting of sequences MH085801 from Banten Indonesia, MH085800 from Gresik Indonesia, MH235639 from Myanmar, KY371466 from China, HQ149843 from Iran, and JF952725 from Japan. Clade II consists of *Cephalopholis* sequences consisting of sequences MN870587 from Ambon Indonesia, KM077909 from New Caledonia, KF009577 from the Philippines, and FJ237607 from India. The phylogenetic tree reconstruction in Figure 2 has a number at the base of the branch called the bootstrap value. The bootstrap value is directly proportional to the confidence level of the phylogenetic reconstruction, the

greater the bootstrap value, the higher the level of confidence in the phylogenetic tree reconstruction that is formed. The bootstrap at the base of the clade serves to show the accuracy of branching phylogenetic trees (Horiike et al. 2009). A phylogenetic tree is a branching system like a tree diagram. Relationships based on DNA sequences or tree-like proteins can be useful for predicting evolutionary processes in the past (Baldauf 2003). Relationships made by phylogenetic analysis can show close genetic distances and can be interpreted in the phylogenetic tree. Reconstruction of phylogenetic trees is supported by the results of genetic distance analysis in a species.

Genetic distance analysis was carried out using MEGA X software. The results of this analysis showed that the BWKR1 *Epinephelus coioides* sample code obtained from Pangpang Bay was not different from the Genbank database from China (KY371466), Banten (MH085801) and Gresik (MH085800) with 0.00 genetic distance. Meanwhile, the sequence from Myanmar (MH235639) has a genetic distance of 0.002, the sequence from Iran (HQ149843) has a genetic distance of 0.006 and from Japan (JF952725) it has a distance of 0.119. In the sample code BWKR2 *Cephalopholis miniata* there is also no difference with the sequences obtained from Genbank Ambon Indonesia (MN870587), New Caledonia (KM077909), Philippines (KF009577). The genetic distance between sequence species also shows the number 0.00-0.02 (Table 1). The type of orange spotted grouper (*Epinephelus coioides*) from Pangpang Bay Banyuwangi compared to sequences from Myanmar, Iran, Banten has a small genetic distance because it is still in the Indian Ocean. In the sequences from China and Gresik, Indonesia has a small genetic distance because it is still in the same Indo-Pacific region. Meanwhile, the sequence from Japan has a genetic distance that is quite far around 0.119. This may occur due to several factors, including the correctness of the data entered in the BLASTN or the presence of contamination which can result in errors in

Table 2. BLASTN results from Genbank NCBI

No.	Sample code	Species	Query Cover (%)	Identity (%)	Reference Sequence GenBank
1.	BWKR1	<i>Epinephelus coioides</i>	100%	100%	KY371466 (China)
2.	BWKR2	<i>Cephalopholis miniata</i>	100%	100%	MN870587 (Ambon, Indonesia)

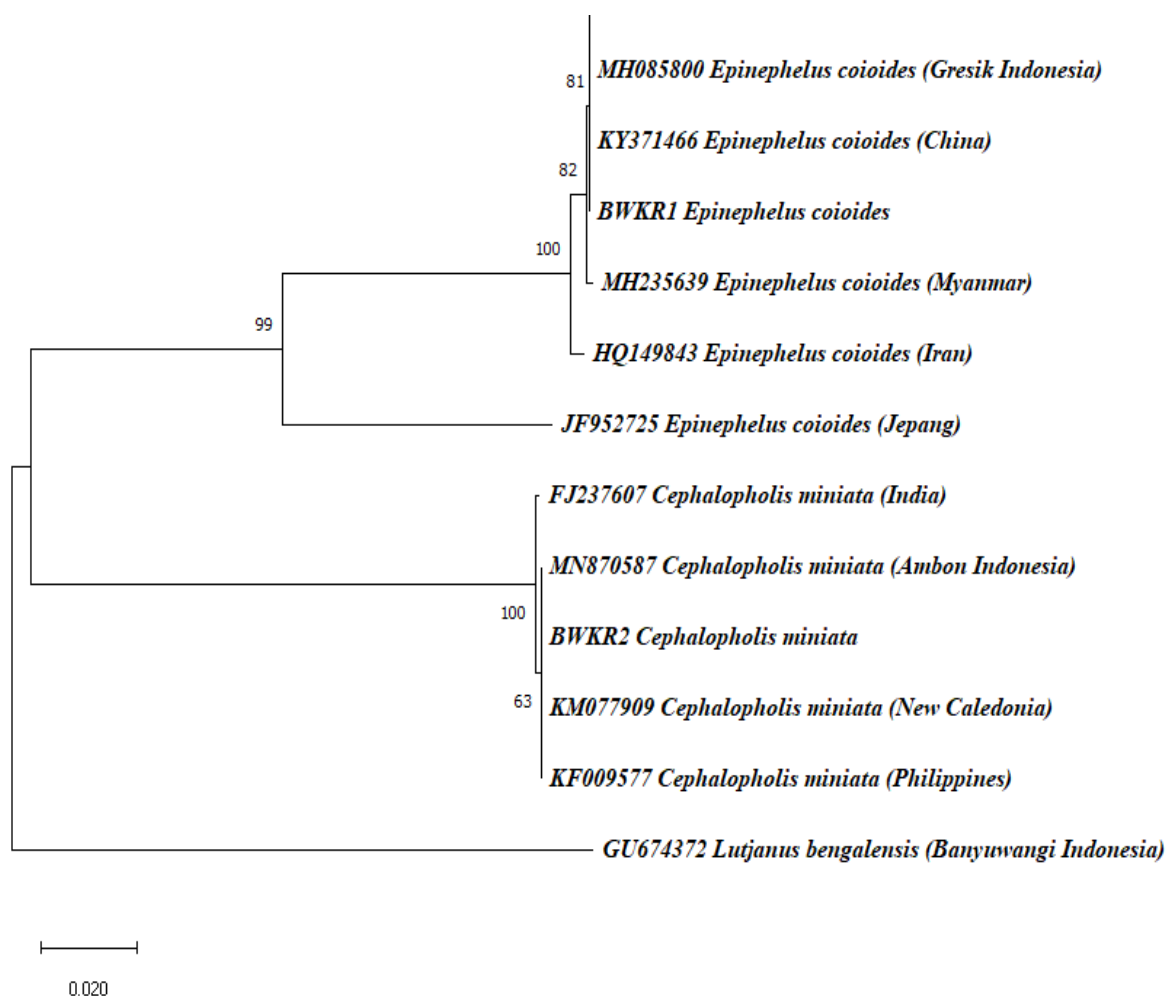


Figure 2. Phylogenetic tree of the sequence sample with sequence from Genbank NCBI

identifying a species. Based on the results of genetic distance red grouper (*Cephalopholis miniata*) from Pangpang Bay has 0.00 genetic distance when compared to sequences from Ambon, Indonesia, New Caledonia, and the Philippines, while the farthest genetic distance is in sequences from India which has a distance of 0.002. Distribution of *Cephalopholis miniata* itself spreads from the Indo-Pacific, Red Sea, South Africa and most islands in the Indian Ocean and the west-central Pacific.

CONCLUSION

Identification of grouper based on molecular identification, it is known that two species of grouper obtained from Pangpang Bay Banyuwangi are *Epinephelus coioides* and *Cephalopholis miniata* according to the database on the NCBI Genbank code KY371466 (China) and MN870587 (Ambon, Indonesia). Morphologically, BWKR1 was identified as *Epinephelus coioides* and BWKR2 was identified as *Cephalopholis miniata*. The

two types of grouper have the same color pattern and meristic characteristics as the morphological identification guide used. The genetic distance between species of *Epinephelus coioides* ranged from 0.00-0.122, while the species of *Cephalopholis miniata* had a genetic distance of 0.00-0.002. In the phylogenetic tree, the grouper *Epinephelus* and *Cephalopholis* form separate clades. This study suggested that it is necessary to conduct research with more samples to see the diversity of grouper DNA and genetic variation of grouper from the Pangpang Bay area of Banyuwangi and other areas (eg : Bali and Nusa Tenggara).

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REFERENCES

- Andriyono, S., & Suciyo, S. 2020. Molecular Identification and Phylogenetic Tree Reconstruction of Marine Fish from the Essential Wetland Area of Banyuwangi, Indonesia. *Egyptian Journal of Aquatic Biology and Fisheries*, 24: 427-439. DOI: 10.21608/ejabf.2020.87032
- Andriyono, S., & Suciyo, S. 2020. Molecular Identification and Phylogenetic Tree Reconstruction of Marine Fish from the Essential Wetland Area of Banyuwangi, Indonesia. *Egyptian Journal of Aquatic Biology and Fisheries*, 24: 427-439. DOI: 10.21608/ejabf.2020.87032
- Baldauf, S.L., 2003. Phylogeny for the faint of heart: a tutorial. *TRENDS in Genetics*, 19(6):345-351. DOI: 10.1016/S0168-9525(03)00112-4
- Folmer, O., Hoeh, W., Black, M., & Vrijenhoek, R. 1994. Conserved primers for PCR amplification of mitochondrial DNA from different invertebrate phyla. *Molecular Marine Biology and Biotechnology*, 3:294-299.
- Horiike, T., Miyata, D., Hamada, K., Saruhashi, S., Shinozawa, T., Kumar, S., Chakraborty, R., Komiyama, T., & Tateno, T. 2009. Phylogenetic construction of 17 bacterial phyla by new method and carefully selected orthologs. *Gene*, 429: 59-64. DOI: 10.1016/j.gene.2008.10.006
- Kamal, M.M., Hakim, A.A., Butet, N.A., Fitrianiingsih, Y., & Astuti, R.J.J.B.T. 2019. Autentikasi spesies ikan kerapu berdasarkan marka gen MT-COI dari perairan Peukan Bada, Aceh. *Jurnal Biologi Tropis*, 19(2):116-123. DOI: 10.29303/jbt.v19i2.1245
- Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Journal Molecular biology evolution* 35(6): p.1547. DOI: 10.1093/molbev/msy096
- Madduppa, H., Ayuningtyas, R.U., Subhan, B., & Arafat, D.J.I. 2016. Exploited but unevaluated: DNA barcoding reveals skates and stingrays (Chordata, Chondrichthyes) species landed in the Indonesian fish market. *Ilmu Kelautan: Indonesia Journal of Marine Science*. 21(2): 77-84. DOI:10.14710/ik.ijms.21.2.77-84
- Morris, A.V., Roberts, C.M., Hawkins, J.P. 2000. The threatened status of groupers (Epinephelinae). *Biodiversity & Conservation*, 9(7):919-942. DOI: 10.1023/A:1008996002822
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, 30(12): 2725-2729. DOI: 10.1093/molbev/mst197
- Zein M.S.A. & Prawiradilaga, D.M. 2013. DNA barcode fauna indonesia: Prenada Media.