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Short Communication

DNA Barcoding of Cardinalfish (Apogonidae) in Gilimanuk Bay, Bali, Indonesia

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Abstract

The Apogonidae is estimated to consist of nearly 300 fish species, most of which inhabit coral reef areas. The lack of distinctive body markings and overlapping species distribution makes species assignment challenging. Therefore, this study aimed to delineate species and establish barcoding reference databases of Apogonidae in Gilimanuk Bay (Bali, Indonesia) using the Cytochrome Oxidase I (COI) gene of the mitochondrial DNA. A total of 22 fish tissue samples were extracted with 10% Chelex solution. BLAST analysis was performed and genetic differentiation between species was calculated. The phylogenetic tree was constructed using the Maximum Likelihood method and tree visualization was generated using iTOL V5. The morphology and genetic identification results based on the mitochondrial COI gene revealed eight species of seven genera, and one species was new to GenBank online database. This study was the first-ever addition of COI sequence for *Ostorhinchus hartzfeldii* into the GenBank database. The average K2P genetic distance within species and K2P distance between genera within the family were 0.60% and 19.10%, respectively. The mean genetic distance between genera within the family was 31.8-fold higher than the mean genetic distance within species. The phylogenetic tree showed that each sample resided in a distinct cluster, which indicates that DNA barcoding is a reliable and effective approach for species delimitation in Apogonidae fishes.

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1. Introduction

Cardinalfish (Apogonidae) is known to have a high number of species (approximately 300 species) which usually inhabit a diverse microhabitat ranging from the water column to coral crevices (Luehrmann *et al*., 2020). Most of these fishes live in association with branching corals (Gardiner and Jones, 2005) and some are strongly associated with sea urchins (Putra and Putra, 2019; Tambunan *et al*., 2022). Cardinalfish are popular among aquarium hobbyists and marine ornamental fish dealers (Vagelli, 2011; Saravanan *et al*., 2013). Indonesia is the second largest country that exported marine fish to the US with a total volume of more than 11 million fish and one of the cardinalfish species (*Pterapogon kauderni*) was on the top list (Rhyne *et al*., 2017). Despite having high species diversity, Apogonidae also shows habitat partitioning, which further drives morphological and molecular differentiation between species (Abecia *et al*., 2018; Sato *et al*., 2021).

Ambiguity in determining species and genera of Apogonidae is relatively high, which indicates the existence of overlapping characters between species (Fraser *et al*., 2002, 2006; Gon and Allen, 2012). The DNA barcoding method's emergence has finally helped validate species, determining genera/subgenera and phylogenetic relationships between species in Apogonidae (Thacker and Roje, 2009; Sato *et al*., 2021). Several new species of Apogonidae were finally discovered, along with the rapid use of DNA barcoding methods (Mabuchi *et al*., 2014; Fraser *et al*., 2021; Lea *et al*., 2022). The application of this method makes the species identification process fast and accurate by utilizing the standard gene for barcoding, namely Cytochrome Oxidase subunit I (COI). This gene is widely used to reveal genetic population structure and differentiate a closely related species across diverse animal phyla ranging from jellyfish (Maas *et al*., 2020), echinoderm (Lessios *et al*., 2003), mollusks (Meyers-Muñoz *et al*., 2016; Keyse *et al*., 2018; Afiati *et al*., 2022), crustacean (Barber *et al*., 2006; Permana *et al*., 2019), sponges (Pöppe *et al*., 2010), fishes (Gon and Allen, 2012; Lea *et al*., 2022; Falah *et al*., 2023), and marine mammals (Alfonsi *et al*., 2013).

Gilimanuk Bay is a narrow and semi-enclosed bay with a depth of about 10 meters which is ecologically unique compared to other coastal areas in Bali (Allen and Erdmann, 2012a). This narrow bay of approximately 37 km^2 is home to 11 mangrove species (Ma'ruf *et al*., 2022), 11 species of seabirds (Pettaloloa *et al*., 2022), four seagrass species (Purnomo *et al*., 2017), 153 coral reef fishes (Allen and Erdmann, 2012a), and various species of corals and invertebrate (Cappenberg *et al*., 2006; Lazuardi *et al*., 2012) that supports more than 300,000 people living around the bay. Despite being proposed as the marine protected area in the western part of Bali due to its biodiversity and habitat complexity (Allen and Erdmann, 2012a), this area is also vulnerable to habitat destruction. Plastics, heavy metals, and a high nitrogen concentration have been known to pollute the bay (Arbi *et al*., 2019; Mbaba *et al*., 2019; Pamungkas *et al*., 2021). Meanwhile, introduced fish, *Pterapogon kauderni* might also compete with native fishes for space or food (Vagelli, 2011), which could further affect the ecosystem services the bay provides. Biodiversity inventory is urgently needed before growing anthropogenic activities and climate change threatens diversity (Delrieu-Trottin *et al*., 2020; Limmon *et al*., 2020).

However, most of Indonesia's barcoding research has mainly focused on commercially important fishes or species of conservation concern. Meanwhile, less effort has been made for small, low economic value and less conservation concern fishes. Hence, the present research will provide the first barcoding data for Apogonidae fishes in Indonesia which could be accessed through GenBank online database. Therefore, this study aims to identify the *Apogon* species and establish a genetic database through the barcoding method using COI of mitochondria.

2. Materials and Methods

2.1 Specimen Collection

A total of 22 specimens of Cardinalfishes were successfully collected during the survey in Gilimanuk bay (Figure 1). Samples were collected from various depths, ranging from 2-7 meters, using scoop nets. A small tissue from each specimen was preserved in 96% ethanol for subsequent analysis. No approval of ethics regarding animal welfare within this study.

2.2 Method

Chelex 10% method was used to extract total DNA from muscle or fin tissues (Walsh *et al*., 1991). The COI gene was amplified with FISH F1 (TCA ACC AAC CAC AAA GAC ATT GGC AC) and FISH R1 (TAG ACT TCT GGG TGG CCA AAG AAT CA) primers (Ward *et al*., 2005). Polymerase Chain Reactions (PCR) were used in 25 µl reactions with a final concentration of 2.5 µl 0.8 mM of dNTPs, 1.25 µl of each primer at 0.5 µM, 2.0 µl 2 mM $MgCl_2$, 14.5 µl ddH_2O, 2.5 µl 10X PCR Buffer, 0.125 µl 2.5 U AmpliTaq (Applied Biosystems),

and 1 µl of DNA template. The thermal cycler was set up with the following profile: a denaturation step of 94°C for 30 seconds, an annealing temperature of 50°C for 30 seconds, extension at 72°C for 45 seconds (this cycle was repeated 38 times) and followed by a final extension of 72°C for five minutes. The amplification products were evaluated on 1% agarose gel stained with GelRed® (Biotium, Inc). Satisfactory products were sent to the 1st Base DNA Sequencing Facility for sequencing processes.

identification. Morphological identification refers to Reef Fishes of the East Indies (Allen and Erdmann, 2012b) as well as the Fishbase online database (https://www.fishbase.se/search.php). Several parameters which indicate the sequence length, number of polymorphic loci, parsimony-informative sites (PIS), and the number of haplotypes were calculated using DnaSP v6 (Rozas *et al*., 2017), while the mean percentage base composition and GC content were calculated in MEGA X. The Kimura-2-parameter (K2P) model (Kimura, 1980) was

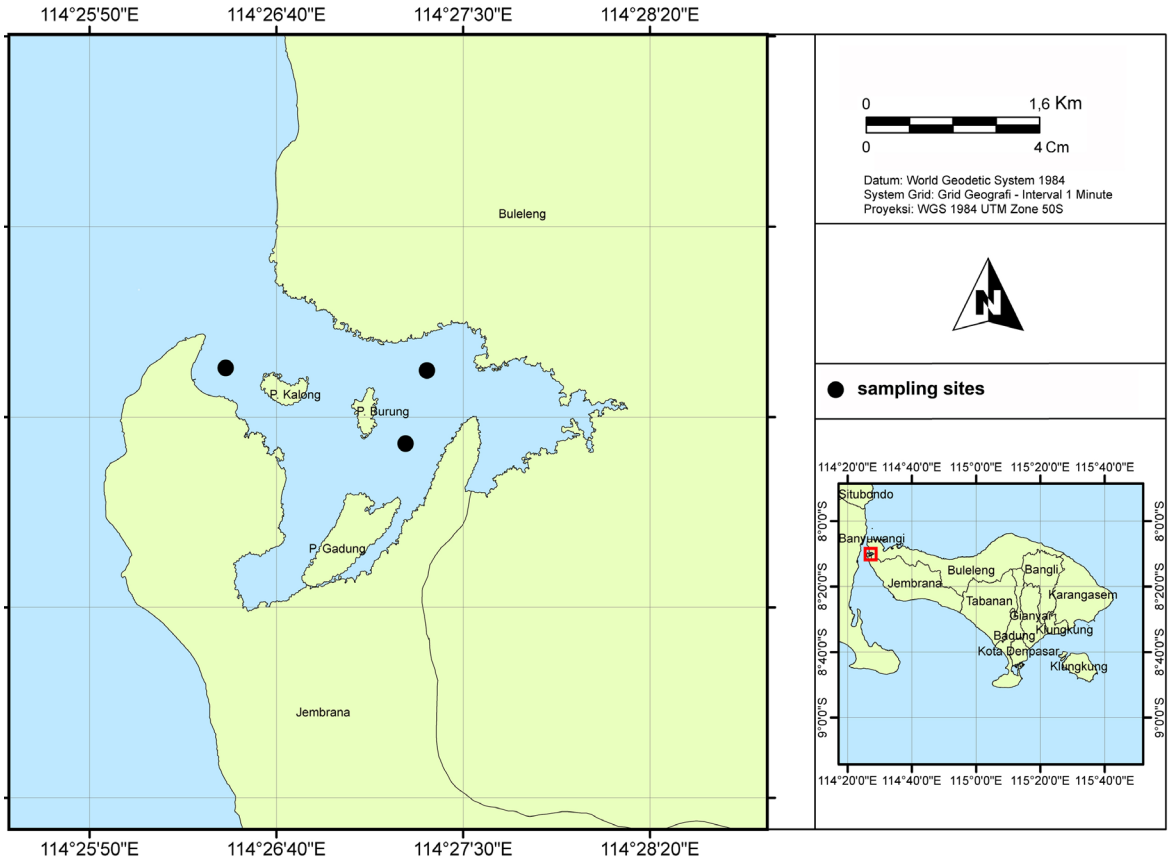

Figure 1. Specimens were collected at three sites within Gilimanuk bay, Bali

2.3 Data Analysis

MEGA X (Kumar *et al*., 2018) was used to edit and visualize the sequences. Sequence alignments were generated using the Clustal W method (Thompson *et al*., 1994) as implemented in MEGA X (Kumar *et al*., 2018). To validate their genetic identity, aligned sequences were uploaded to GenBank using BLAST (Basic Local Alignment Search Tools) in NCBI (http://www.ncbi.nlm.nih.gov/BLAST) with a similarity threshold of over 98% for all sequences (Arroyave and Stiassny, 2014). The specimens will be reexamined if there is any discrepancy between barcoding results and morphological calculated using MEGA X to determine the genetic differentiation within and between species.

To generate a phylogenetic analysis of the cardinalfishes, an analysis of Maximum Likelihood (ML) was performed. The Maximum likelihood is a robust and most commonly used method which uses an explicit evolutionary model (Truszkowski & Goldman, 2016; Delrieu-Trottin *et al*., 2020). ML analysis was performed with the software RaxMLGUI 2.0 (Silvestro and Michalak, 2012; Stamatakis, 2014) using the Hasegawa-Kishino-Yano substitution model (HKY) with gamma distribution (+G) and invariable

evolutionary sites (+I), and bootstrap analysis of 1000 replicates. This substitution model was determined under the Bayesian Information Criterion (BIC) in MEGA X. According to BIC, HKY+G+I is the best substitution model because it contains the lowest BIC scores (Nei and Kumar, 2000). The consensus tree was visualized using the iTOL V5 (Letunic and Bork, 2021; http://itol.embl.de/).

pairs with average nucleotide composition of A (22.7%), T (28.6%), G (19.5%), and C (29.2%) (Table 2). The mean C content was the highest, while G content was the lowest. The GC content (48.7%) was lower than AT content (51.3%). The GC content decreased in the first codon but increased in the second and decreased again in the third codon with a mean value of 23.5%, 25.3%, and 24.3%, respectively.

Table 1. Specimens collected from Gilimanuk bay with sample Id, species name, number of specimens (n) and GenBank accession numbers

No.	Sample Id	Species	n	Accession number
1	BIOSUB160_001; BIOSUB160_002; BIOSUB160_003.	*Sphaeramia nematoptera*	3	OQ341520; OQ341521; OQ341522
2	BIOSUB160_004; BIOSUB160_005; BIOSUB160_006	*Cheilodipterus quinquelineatus*	3	OQ341523; OQ341524; OQ341525
3	BIOSUB160_007	*Ostorhinchus hartzfeldii*	1	OQ341526
4	BIOSUB160_008; BIOSUB160_009 BIOSUB160_010	*Fibramia thermalis*	3	OQ341527; OQ341528; OQ341529
5	BIOSUB160_011; BIOSUB160_012; BIOSUB160_013	*Pterapogon kauderni*	3	OQ341530; OQ341531; OQ341532
6	BIOSUB160_014; BIOSUB160_015; BIOSUB160_016	*Zoramia leptacantha*	3	OQ341533; OQ341534; OQ341535
7	BIOSUB160_017; BIOSUB160_018; BIOSUB160_019	*Rhabdamia gracilis*	3	OQ341536; OQ341537; OQ341538
8	BIOSUB160_020; BIOSUB160_021; BIOSUB160_022	*Ostorhinchus hoevenii*	3	OQ341539; OQ341540; OQ341541

3. Results and Discussion

3.1 Results

According to morphological and molecular analysis, this study revealed eight species of seven genera. All sequences obtained in this study have been uploaded to GenBank online database with accession numbers: OQ341520-OQ341541 (Table 1). Among eight species of fish, one was new to GenBank online database (*Ostorhinchus hartzfeldii;* accession number OQ341526). The sequence read lengths were 677 base

The analysis of nucleotide pair frequency showed that 451 of 677 (66.62%) sites were conserved, 226 of 677 (33.38%) sites were variable, 218 of 677 (32.20%) sites were parsimony informative, and 8 of 677 (1.18%) sites were singleton. A total of 18 haplotypes were successfully generated, and the value of haplotype diversity (Hd) was 0.98. The mean genetic distance (K2P distance) within species was 0.60% with a maximum value of 1.70%, while the mean K2P distance between genera within the family was 16.77% with a maximum value of 21.51 (Table 3). The mean

genetic distance between genera within the family was 31.8-fold higher than the mean genetic distance within species.

The Maximum Likelihood (ML) tree was built, including 22 sequences, and eight sequences were retrieved from GenBank online database (Accession number: EU398997, AB890068, AB890099, MN733703, FJ583995, FJ584119, KP194998, and MN733539). All sequences from the same species belonged to single distinct clusters with bootstrap values of more than 80, except for BIOSUB160 007, represented by only one specimen (Figure 2). According to BLAST, this specimen was identified as *Ostorhinchus septemstriatus* with a percent similarity of 87.27%. Since the value was lower than 98%, the specimen's morphology was reexamined and compared to *O. septemstriatus.* The most distinctive characteristic differentiating these fishes was the absence of a black spot at the edge of the caudal peduncle in *O. septemstriatus.* Therefore, it can be concluded that the specimen with the ID BIOSUB160 007 was not *O. septemstriatus.* After comparing with the literature (Allen and Erdmann, 2012b) and Fishbase online database, this specimen was identified as *Ostorhinchus hartzfeldii* . No nucleotide data were available in GenBank, nor did the BOLD system refer to *O. hartzfeldii.* Therefore, this is the first study that provided nucleotide sequence of *O. hartzfeldii* in GenBank database.

3.2 Discussion

The base composition analysis of AT content (51.3%) was higher than GC content (48.7%), which is supported by previous studies conducted in Taiwan Strait (Bingpeng *et al*., 2018) and Aceh, Indonesia (Fadli *et al*., 2020). The mean interspecific distance value was 31.8-fold higher than the mean genetic distance within species. This result was congruent to the 31-fold higher difference observed in Taiwan marine fishes (Bingpeng *et al*., 2018), the 30-fold higher observed in Ambon (Limmon *et al*., 2020), and the 46.5-fold higher difference in reef fishes of Aceh, Indonesia (Fadli *et al*., 2020). This also confirms that interspecific distance sufficiently outscores intraspecific distance.

The average K2P distance within species and between genera within the family were 0.60% and 19.10%, respectively. Numerous fish barcoding studies have found similar patterns to the present study.

Table 2. The nucleotide base composition percentage (ATGC) and GC content percentage of the first, second and third codon positions; Min (minimum value), Max (maximum value), SE (standard error)

Nucleotide content	Mean	Min.	Max.	SE
A%	22.7	20.7	24.2	0.199
T%	28.6	24.8	31.7	0.461
G%	19.5	17.9	21.0	0.200
C%	29.2	26.8	31.9	0.366
GC% 1st codon	23.5	22.3	25.7	0.207
GC% 2nd codon	25.3	21.2	28.8	0.594
GC% 3rd codon	24.3	23.8	25.3	0.081

Table 3. Comparisons of genetic differentiation (percent of K2P distance) within species and genus levels; Min (minimum value), Max (maximum value), SE (standard error)

Comparisons within	Taxa	Mean (%)	Min. (%)	Max. (%)	SE
Species	8	0.60	0.10	1.70	0.002
Family	1	19.10	16.77	21.51	0.018

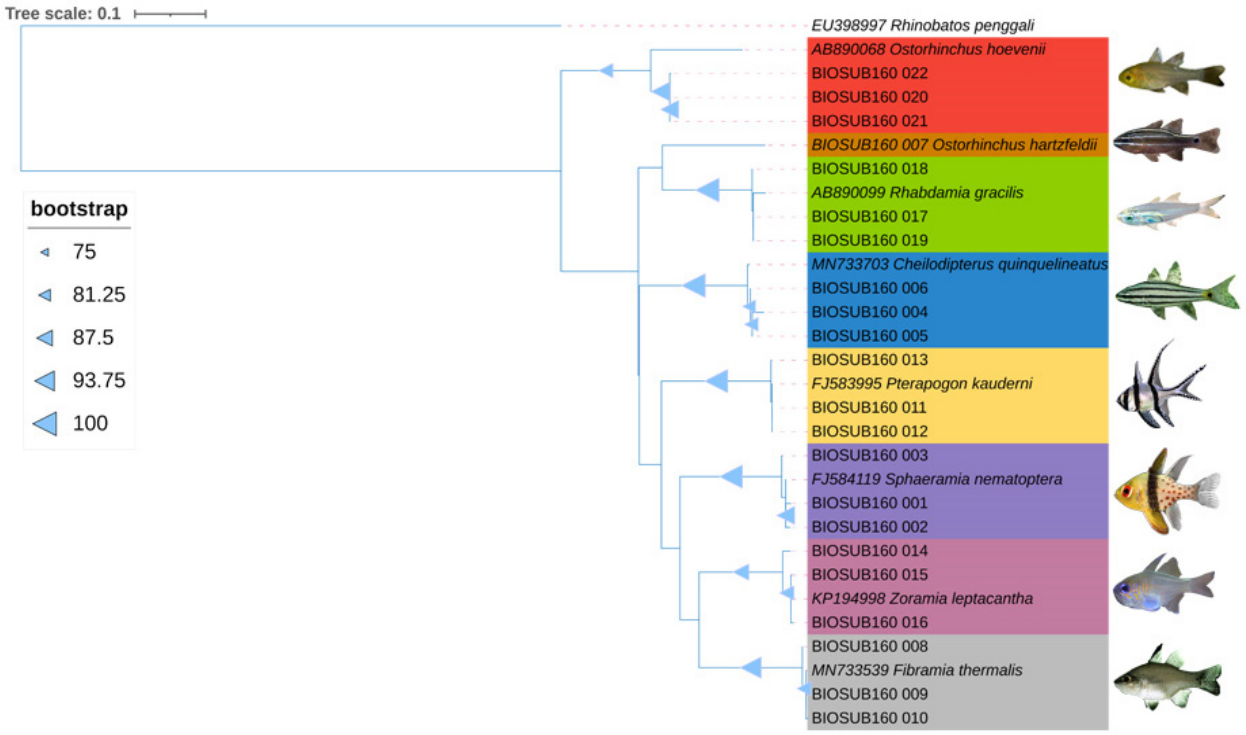

Figure 2. The Maximum Likelihood tree of Apogonidae in Gilimanuk bay was constructed under HKY+G+I model. The bootstrap values shown were more than 75%. Tree visualization was generated using an online Interactive Tree of Life (ITOL, https://itol.embl.de/).

For instance, the mean K2P distances within species and family were 0.21% and 21.30%, respectively, in Rongcheng Bay, China (Wang *et al*., 2018); the average values of 0.21% (within species) and 23.70% (within the family) in Taiwan Strait (Bingpeng *et al*., 2018); the average values of ray-finned fishes of Vietnam were 0.34% (conspecifics) and 17.39% (confamilial) (Thu *et al*., 2019); the genetic distance within species and family were found 0.24% and 19.01% respectively in Aceh, Indonesia (Fadli *et al*., 2020); and the values of fishes in Ambon bay were 0.32% within species and 18.28% within the family (Limmon *et al*., 2020). Commonly, a high value of average genetic distance was observed at higher taxonomic levels (Wang *et al*., 2018; Thu *et al*., 2019).

Although debatable, K2P distance remains a standard model to aid species delimitation (Reid *et al*., 2011). Therefore, the K2P model was used to ensure that the results of this study can be compared to other barcoding studies. Species that showed intraspecific variation above 2% were assumed to be different species (Thu *et al*., 2019). The average genetic distance within species in this present study was below 2%, which means that each species could be differentiated clearly and using barcoding to delineate Apogonidae species is feasible (Table 3).

ML tree showed that each species of Apogonidae was clustered into a single monophyly clade without any overlap between species. This result confirms that the barcoding method is effective and reliable in determining the species level. Several barcoding studies had been successfully assigned marine fishes to their species level, and describing new species (Landi *et al*., 2014; Fraser *et al*., 2021; Lea *et al*., 2022), but other studies also noticed that the method has limited ability to determine the higher taxa (e.g., genera and family) (Landi *et al*., 2014; Thu *et al*., 2019). Recent studies have shown that fish biodiversity is under threat caused by overfishing, habitat destruction, and climate change (Cinner and Mcclanahan, 2006; Alvarenga *et al*., 2021; Sultana *et al*., 2022). Moreover, coral reef destruction could decline the global reef fish biodiversity by around a half if corals are lost (Strona *et al*., 2021). Therefore, providing reliable and accessible data, either morphology or genetic, was the first important thing to do while managing conservation plans.

4. Conclusion

The present study revealed that the barcoding method effectively and reliably delineates Apogonidae at the species level. Thus, DNA barcoding is the complement for morphological identification. The

present study successfully identified eight species of seven genera; one was new to GenBank online database. This study's results could be further implemented to manage fisheries activities and evaluate fish biodiversity in Gilimanuk Bay, Bali.

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Authors' Contributions

The contribution of each author is as follows, INGP; established idea, conceptualized, provided funding acquisition, collected data, wrote manuscript, and analyzed data. GSR and EF; collected the data and revised the manuscripts. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

All authors declared no conflict of interest upon the publication of this manuscript.

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