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MOLECULAR SPECIES IDENTIFICATION OF ROCKSKIPPER (PISCES: BLENNIIDAE) FROM POROK BEACH (YOGYAKARTA, INDONESIA) BASED ON *16S rRNA* and *COI* GENES

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Rockskipper belongs to the species-rich family of Blenniidae (*Combtooth blenny*) and is known for its cryptic and species complexes presence. Identifying cryptic species based solely on morphological features is challenging due to their similar morphological characteristics. As a result, molecular genetic techniques based on two partial mtDNA genes, *COI* and *16S* rRNA, were employed to identify fish accurately. This study aimed to evaluate the effectiveness of *COI* and *16S rRNA* gene for the identification of Rockskipper fish and investigate the genetic relationship between species of Rockskipper from Porok Beach. The result revealed that the thirteen Rockskipper samples from Porok Beach that belong to six species (*E. vermiculatus, E. striatus, I. lineatus, I. dussumieri, I. edentulus,* and *B. caudolineata*) with more than 99% similarity. In contrast, the *16S* analysis identified five species. A Bayesian phylogenetic tree demonstrated that six species of Rockskipper from Porok Beach are genetically distinct and separated into two clusters. We also found that two samples (RS-9 and RS-10) form a monophyletic group with *B. caudolineata* with maximum bootstrap (NJ and ML: 100%) and posterior probability (1.00). We hypothesized that *B. caudolineata* is a species complex with at least two lineages: one was genetically closer to RS-8, and another was more closely related to RS-9 and RS-10. Both the *COI* and *16S* rRNA genes were found to be capable of delineating species and revealing genetic variation among Rockskipper samples in this study. However, our findings demonstrated that the *COI* gene is a more accurate and

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reliable marker for identifying Rockskipper species from Porok Beach, Gunungkidul, Yogyakarta, Indonesia.

Key words: 16S rRNA, *COI*, Porok Beach, Rockskipper

INTRODUCTION

Rockskipper belongs to the family Blenniidae (Combtooth blenny), which includes 59 genera and 405 species (FRICKE *et al*., 2022). The Blenniidae is a species-rich family of marine fishes and is rarely found in fresh or brackish water. These fishes are widely distributed in the Atlantic, Indian, and Pacific oceans (NELSON, 2016). The term "Combtooth blenny" refers to the peculiar teeth of these fish, characterized by a row of comb-shaped teeth on the dentaries and premaxillaries, as well as the absence of teeth on other oral cavity bones (except for certain species, which have teeth on the vomer) (SPRINGER, 1968; HASTING and SPRINGER, 2009).

For many years, the family Blenniidae was classified primarily based on morphological characteristics, even though the family includes taxonomic gaps, misidentification, and mitochondrial introgression (PHAM *et al*., 2022). Furthermore, fish of the family Blenniidae have been known for the presence of cryptic and species complexes, confirmed using molecular markers. For example, *Omobranchus punctatus* comprised five species (*O. punctatus sensu stricto*, *O. dispar*, *O. sewalli*, *O.* cf. *kochi*, and *O.* cf. *japonicus*) based on morphological characteristics, then genetically confirmed using mitochondrial (*COI*) and nuclear markers (*ENC1*, *myh6*, *sreb2*, and *tbr1*) (GIBBS *et al*., 2018; CABEZAS *et al*., 2022). Other examples, *Cirripectes alboapicalis*, consisted of at least three cryptic species that were confirmed by *COI* mtDNA (DELRIEU-TROTTIN *et al*., 2018), and *Scartella cristata*, a species complex with five verified clades based on mtDNA *D-loop* (ARAUJO *et al*., 2020).

Due to their similar morphological characteristics, identifying cryptic species based solely only on morphological features is challenging, and incorrect identification of cryptic species could contribute to significant taxonomic and conservation issues. Therefore, molecular genetic approaches employing mitochondrial DNA (mtDNA) for species identification have been used and developed in recent years. The mtDNA *COI* (Cytochrome Oxidase subunit I) gene has been widely accepted as a reliable, universal animal species-level barcode for the majority of the animal kingdom, including fishes (HEBERT *et al*., 2003b; NGILI *et al*., 2015; PENINAL *et al*., 2017; SYAIFUDDIN *et al*., 2017; BINGPENG *et al*., 2018; LINH *et al*., 2018 RHA'IFA *et al*., 2021; AJI and ARISURYANTI, 2021). One of the main objectives of DNA barcoding is to support the rapid identification of potential unknown species, especially cryptic species using one or a few short, standardized DNA regions (HEBERT *et al*., 2003a; BICKFORD *et al*., 2007). The *16S* rRNA gene has been used for identification due to its highly conserved among the animal taxa (VENCES *et al*., 2005). The combination of both *COI* and *16S* rRNA was also the prominent marker for the identification of different fishes, such as Nigerian freshwater catfish and Tilapia (FALADE *et al*., 2016), kissing gourami (ARISURYANTI *et al*., 2019), deep-sea eels (KIM *et al*., 2021), and brackish and marine water fishes (HABIB *et al*., 2021).

Porok Beach is located in Kemadang Village, Tanjungsari Subdistrict, Gunungkidul District, Yogyakarta, Java Island, Indonesia. Porok Beach is one of the beaches in Gunungkidul that have a large diversity of fish species, including Rockskipper fish. This fish is particularly found in the intertidal zone (UCHIYAMA, 2012; WHITE *et al*., 2015). A previous study was

conducted to determine the diversity of Rockskipper fish on this beach (AZIZAH *et al*., 2021). However, there is no molecular genetic study of Rockskipper has been conducted in this location. Therefore, the objective of the present study was to evaluate the effectiveness of *COI* and *16S* rRNA gene for the identification of Rockskipper fish and investigate the genetic relationship between species of Rockskipper from Porok Beach, Yogyakarta, Indonesia.

MATERIALS AND METHODS

Sample collection

Thirteen samples of Rockskipper fish (code: RS-01 to RS-13) were collected from Porok Beach, Gunungkidul, Yogyakarta with grid references 8°8'1" S 110°33'16" E (Figure 1). The sampling stations were at the same location, and all samples were collected simultaneously using a hand net (Figure 2). Muscle tissue from each fish was meticulously excised and placed into separate sterile 1.5 ml tubes filled with 99% ethanol for long-term preservation. Following collection, both the preserved muscle tissue and the entire fish specimens were transported to the Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada (Yogyakarta, Indonesia), where they were stored at -20ºC for subsequent analysis.

Figure 1. Map location of Rockskipper fish collected from Porok Beach.

Figure 2. Thirteen Rockskipper fish collected from Porok Beach (Yogyakarta, Indonesia). Bar scale = 1 cm.

DNA isolation, amplification, and sequencing

Total genomic DNA from each muscle fish (50-100 mg) was extracted using DNeasy blood and tissue kit (QIAGEN, Valencia, CA, USA) following the manufacturer's protocols. The total dilution volume of genomic DNA of each Rockskipper fish was 250 µL. A total of two partial mitochondrial genes of *COI* and *16S* rRNA were amplified in this study. Primers used for the amplification of the *COI* mitochondrial region were FishF2 (5'-TCG ACTAATCATAAAGATATCGGCAC-3') and FishR2 (5'-ACTTCAGGGTGACCGA AGAATCAGAA-3') (WARD *et al*., 2005), and whereas *16S* rRNA region was amplified using two primers, 16Sar (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sbr (5'-CCGGTCTGAACTCAGATCACGT-3') (PALUMBI, 1996). PCR reactions for each gene were carried out in 25 µL volume containing 12.5 µL MyTaq HS Red Mix PCR Kit (Bioline), 10-100 ng of genomic DNA, 2 mM $MgCl_2$, 0.6 µM of each primer, and 5.5 µL ddH_2O in a Thermal Cycler (Biorad). PCR conditions for both genes involved an initial denaturation at 95ºC for 2 min, then 35 cycles of 95ºC for 15 s, 50ºC for 30 s, 72ºC for 30 s, and a final extension of 72ºC for 5 min (ARISURYANTI *et al*., 2020b). The electrophoresis of PCR products was performed on a 1 % agarose gel stained with Florosafe (Bioline) and buffered with Tris-acetate EDTA (TAE) at 100 volts for 15 minutes. The visualization was done with ultraviolet light. The amplification samples of the *COI* gene were then transported to 1st Base Sdn Bhd. (Malaysia) through P.T. Genetika Science (Jakarta), whereas *16S* amplification samples were sent to LPPT UGM for DNA purification and sequencing.

Sequence and Phylogenetic analysis

DNA sequences result of both genes (*COI* and *16S* rRNA) were inspected and edited manually on the GeneStudio program, then validated using SeqMan and EditSeq in Lasergene DNASTAR software package (DNASTAR Inc., Madison, USA). Chromatograms from forward and reverse primers were inspected for noisy and ambiguous base calling, and noisy tails were trimmed to perform consensus fragments. The *COI* consensus sequence of each Rockskipper fish sample was then translated into amino acids to check the stop codon using vertebrate mitochondrial code. The *COI* consensus sequencing results were examined using the Nucleotide BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and BOLD (https://www.boldsystems.org/index.php/IDS_OpenIdEngine), while *16S* rRNA consensus sequence only examined by BLAST. The results of this analysis were used to verify the species of Rockskipper fish by comparing the similarity and query cover to data in GenBank and BOLD. Both sequences were then converted into fasta format and aligned using opal in MESQUITE ver. 3.51 package (MADISON and MADISON, 2018) and ClustalW in MEGAX (KUMAR *et al*., 2018).

The composition of mtDNA nucleotide was calculated with the MEGAX program. The intrapopulation genetic variation analyses including the number of haplotypes, number of polymorphic sites, haplotype diversity, and nucleotide diversity were calculated using DnaSP ver. 6.0 program (ROZAS *et al*., 2017). Kimura-2-parameter (K2P) model was used to estimate intrapopulation and interspecific genetic distance (HEBERT *et al*., 2003a). Twelve *COI* sequences and eight *16S* rRNA sequences of Rockskipper fish i.e *I. lineatus* (*COI*: KX301854 and KX301854; *16S*: KX301940 and KX301941), *I. dussumieri* (*COI*: JF493681 and KX301859; *16S*: KX301942), *I. edentulus* (*COI*: KP194586 and JF493684), *B. caudolineata* (*COI*:

KX301834 and KX301834; *16S*: KX301917 and KX301918), *E. vermiculatus* (*COI*: MT111612 and KX301879; *16S*: KX301962), and *E. striatus* (*COI*: MT111613 and KX301868; *16S*: KX301947 and KX301952) were taken from GenBank (www.ncbi.nlm.nih.gov) and used for comparative purposes whereas whole genome mitochondrial sequence of *Eptatretus burgeri* (AJ278504) was used as an outgroup for phylogenetic analysis of both genes.

The phylogenetic tree was estimated using the Neighbor-Joining and Maximum Likelihood methods with 1000 bootstraps using on MEGAX program (KUMAR *et al*., 2018) and Bayesian Inference on the BEAST program (SUCHARD *et al*., 2018) for each gene and the combination of both genes. The Akaike Information Criterion (AIC) implemented in jModelTest 2.1.10 (DARRIBA *et al*., 2012) was used to determine the best fit evolutionary model. The most optimal sequence substitution models for the Bayesian phylogenetic tree are HKY with invariant sites and gamma (HKY+I+G) for *COI*, GTR with gamma (GTR + G) for *16S*, and both combination of *COI + 16S* on the Akaike Information Criterion (AIC). The Markov chain Monte Carlo (MCMC) was run for 10^7 generations to estimate the posterior probabilities distribution with a sampling frequency set to every 1000 generations. The analysis used a relative burn-in of 25% for diagnostics. The consensus trees were visualized in FigTree 1.4.4 (RAMBAUT, 2019).

RESULTS

Species Verification (COI and 16S rRNA)

Thirteen *COI* consensus sequences of Rockskipper fish generated fragment lengths ranging from 708-711 bp, translated into 236-237 amino acids. There were no stop codons, gaps, or insertions/deletions in sequences. The Nucleotide BLAST and BOLD analyses revealed that the 13 Rockskipper samples belong to six different species: five samples of *Entomacrodus vermiculatus* (code: RS-01, RS-02, RS-03, RS-04, and RS-05 with 99.69-99.70% similarity), one sample of *Entomacrodus striatus* (code: RS-08 with 99.85-99.55% similarity), two samples of *Istiblennius lineatus* (code: RS-06 and RS-13 with 100% similarity), one sample of *Istiblennius edentulus* (code: RS-07 with 100% similarity), one sample *Istiblennius dussumieri* (code: RS-12 with 100% similarity), one sample *Blenniella caudolineata* (code: RS-08 with 99.54% similarity), and two samples of unverified species (RS-9 and RS-10) with low similarity (93.43%) to *Blenniella caudolineata* from GenBank. In this study, we treated and grouped those two samples to *Blenniella caudolineata.* In contrast, thirteen *16S* rRNA consensus sequences of Rockskipper fish with fragment lengths ranging from 617–648 bp only verified five species with two samples (RS-6 and RS-7), revealing different species compared to the *COI* result. The two unverified samples (RS-9 and RS-10) were also detected with low similarity (94.70%) to *Blenniella caudolineata* from GenBank. We only employed *COI* results for species verification since *COI* is more reliable in identifying species as a DNA barcoding marker.

COI sequence analysis

The alignment of thirteen Rockskipper *COI* sequence samples from Porok Beach yielded a clean sequence of 678 bp. The *COI* sequences showed no similar nucleotide composition, except for RS-1 to RS-5 and RS-9 with RS-10. The nucleotide differences between samples were 0-3.39% (T), 0-4.28% (C), 0-2.95% (A), and 0-2.21% (G) (Table 1).

Table 1. Nucleotide composition percentage of Rockskipper fish collected from Porok Beach based on COI mtDNA

Samples	T	C	A	G	A+T	G+C
RS-01	30,38	27,88	22,71	19,03	53,10	46,90
RS-02	30,38	27,88	22,71	19,03	53,10	46,90
RS-03	30,38	27,88	22,71	19,03	53,10	46,90
RS-04	30,38	27,88	22,71	19,03	53,10	46,90
RS-05	30,38	27,88	22,71	19,03	53,10	46,90
RS-06	29,50	28,76	22,57	19,17	52,06	47,94
RS-07	29,20	29,20	22,57	19,03	51,77	48,23
RS-08	30,97	27,29	23,30	18,44	54,28	45,72
RS-09	30,68	27,14	23,60	18,58	54,28	45,72
RS-10	30,68	27,14	23,60	18,58	54,28	45,72
RS-11	32,60	24,93	25,52	16,96	58,11	41,89
RS-12	29,50	28,61	23,16	18,73	52,65	47,35
RS-13	29,50	28,76	22,86	18,88	52,36	47,64

Table 2. Kimura 2-parameter intrapopulation and interspesific genetic distance (%) of six species of Rockskipper fish based on COI mtDNA

	E. vermiculatus	*I. lineatus*	*I. edentulus*	*B. caudolineata*	*E. striatus*	*I. dussumieri*
E. vermiculatus	0,00 (0.00-0.00)					
I. lineatus	21.61 (21.50-21.71)	0.00-0.30				
I. edentulus	21.96 (21.96)	19.87 (1.76-2.84)	0.00 (0.00-0.00)			
B. caudolineata	20.92 (20.74-21.01)	20.63 (19.75-20.97)	20.24 (20.12-20.48)	0.00-11.81		
E. striatus	19.73 (19.73)	20.83 (20.72-20.93)	23.00 (23.00)	20.55 (20.24-20.71)	0.00 (0.00-0.00)	
I. dussumieri	23.61 (23.61)	17.35 (17.26-17.45)	20.00 (20.00)	19.52 (17.75-20.41)	21.54 (21.54)	

The intrapopulation K2P genetic divergence ranged from 0-11.81%. This high genetic divergence (11.81%) was observed between two unverified samples (RS-9 and RS-10) compared to *B. caudolineata* (RS-08). The highest K2P interspecies genetic divergence was between *E.*

vermiculatus and *I. dussumieri* (23.61%). Moreover, the lowest value was between *I. lineatus* and *I. dussumieri* (17.35%). The pairwise genetic distance values of intraspecies and interspecies (K2P) based on *COI* sequences are given in Table 2.

The phylogenetic tree topology of the NJ, ML, and BI trees was almost identical. Therefore, only the Bayesian tree was displayed in this study (Figure 3). Two major clusters were obtained in this analysis. The first cluster consisted of *I. lineatus*, *I. dussumieri*, *I. edentulus*, and *B. caudolineata*, whereas the second cluster consisted of *E. vermiculatus* and *E. striatus*. Each Rockskipper sample from Porok Beach clustered together forming monophyletic clades with *COI* sequences from GenBank and BOLD based on their respective species (visualized in different colors). The RS-9 and RS-10 also formed a monophyletic clade (grey color) with the *B. caudolineata* clade. In addition, *B. caudolineata* (KX301835) was genetically closer to RS-9 and RS-10 than another sample of *B. caudolineata* (KX301834) (that close to RS-8). All these clade formations were supported with high bootstraps (NJ and ML: 99-100%) and posterior probability values (1.00).

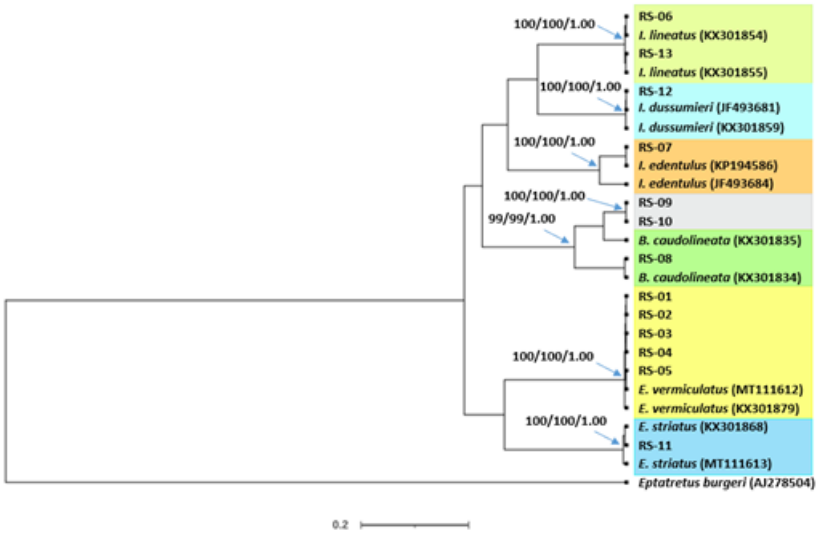

Figure 3. Bayesian phylogenetic tree inferred from mitochondrial *COI* sequences. The tree was produced from 10^7 generations using the HKY+I+G model. The number of each node represents posterior probabilities and the scale corresponds to substitutions/site

The intrapopulation genetic variation revealed no variation in five samples of *E. vermiculatus* (RS-1 to RS-5). On the other hand, the genetic variation was observed in two *I. lineatus* (RS-06 and RS 13). Two haplotypes and two variable sites (sites 285 and 504) were detected in 681 bp *COI* sequences of *I. lineatus*. Both sites were detected as transition pairs. The nucleotide diversity and haplotype diversity were 0.00294±0.00147 and 1.00±0.50, respectively.

The intrapopulation genetic variation was also observed in *B. caudolineata* (RS-8) compared with RS-9 and RS-10. From 684 bp of those three *COI* sequences (RS-8, RS-9, and RS-10), two haplotypes were detected with 73 polymorphic sites. The site positions were 21, 25, 30, 42, 45, 51, 54, 57, 63, 69, 72, 75, 90, 102, 105, 111, 114, 129, 150, 153, 156, 159, 165, 180, 189, 195, 198, 219, 240, 246, 249, 252, 288, 291, 295, 321, 327, 342, 357, 360, 373, 375, 384, 393, 396, 399, 421, 453, 456, 465, 492, 498, 501, 504, 516, 522, 525, 528, 546, 549, 558, 564, 567, 571, 585, 594, 597, 612, 618, 624, 639, 651, and 657. The transition pairs (si=54) were more frequent than transversion pairs (sv=19). The nucleotide diversity and haplotype diversity were 0.07115±0.03354 and 0.667±0.314 respectively.

16S rRNA sequence analysis

A total of thirteen Rockskipper *16S* rRNA sequence samples from Porok Beach were aligned and produced a clean sequence of 596-613 bp. Gaps/insertions/deletions were observed in all thirteen *16S* rRNA sequences. The differences nucleotide between samples were 0-3.82% (T), 0-2.10% (C), 0-1.53% (A), and 0-1.59% (G) (Table 3).

Table 3. Nucleotide composition percentage of Rockskipper fish collected from Porok Beach based on 16S rRNA mtDNA

Samples	T	C	A	G	A+T	G+C
RS-01	23,75	24,58	29,77	21,91	53,51	46,49
RS-02	23,75	24,58	29,77	21,91	53,51	46,49
RS-03	23,58	24,75	29,77	21,91	53,34	46,66
RS-04	23,75	24,58	29,77	21,91	53,51	46,49
RS-05	23,75	24,58	29,77	21,91	53,51	46,49
RS-06	23,75	24,58	29,77	21,91	53,51	46,49
RS-07	21,14	26,68	30,20	21,98	51,34	48,66
RS-08	23,33	25,00	28,67	23,00	52,00	48,00
RS-09	22,28	25,46	28,98	23,28	51,26	48,74
RS-10	22,28	25,46	28,98	23,28	51,26	48,74
RS-11	24,96	24,63	28,71	21,70	53,67	46,33
RS-12	23,28	25,29	29,15	22,28	52,43	47,57
RS-13	21,14	26,68	30,03	22,15	51,17	48,83

The intrapopulation K2P genetic divergence ranged from 0-9.27%. The mean genetic divergence of two unverified samples (RS-9 and RS-10) compared to *B. caudolineata* (RS-08) was 7.90%. RS-06 (recognized as *E. vermiculatus* in *16S* rRNA BLAST analysis) had a genetic divergence of 9.27% compared to *I. lineatus*, the verified species for RS-06 in *COI* analysis. The highest K2P interspecies genetic divergence was observed between *E. striatus* and *I. dussumieri* (20.00%), and the lowest value was between *I. edentulus* and *I. lineatus* (4.62%). The pairwise genetic distance values of intraspecies and interspecies (K2P) based on *16S rRNA* sequences are presented in Table 4.

Table 4. Kimura 2-parameter intrapopulation and interspesific genetic distance (%) of six species of Rockskipper fish based on 16S mtDNA

	E. vermiculatus	*I. lineatus*	*I. edentulus*	*B. caudolineata*	*E. striatus*	*I. dussumieri*
E. vermiculatus	0,17 (0.00-0.17)					
I. lineatus	9.27 (0.00-9.27)	0.00-9.27				
I. edentulus	9.03 (8.87-9.07)	4.62 (0.17-9.07)	0.00 (0.00-0.00)			
B. caudolineata	10.24 (9.85-11.16)	11.90 (9.85-13.61)	13.30 (13.11-13.39)	(0.00-7.90)		
E. striatus	15.38 (15.20-15.42)	15.83 (15.42-16.24)	16.46 (16.46)	17.87 (17.83-17.96)	0.00 (0.00-0.00)	
I. dussumieri	11.51 (11.35-11.56)	10.11 (8.66-11.56)	8.47 (8.47)	15.68 (15.57-15.91)	20.00 (20.00)	0.00 (0.00-0.00)

Two large clusters were generated in this phylogenetic tree (Figure 4) and differ from the *COI* tree result. The first cluster consisted of *E. vermiculatus*, *I. lineatus*, *I. dussumieri*, and *B. caudolineata*. The second cluster only included *E. striatus*, which serves as the basal taxon for all Rockskipper species in this study. Similar to the *COI* tree, each Rockskipper sample from Porok Beach formed monophyletic clades with *16S* rRNA sequences of Rockskipper from GenBank based on their respective species (shown in different colors) except for the RS-06 and RS-07. Due to mismatched sample identification between *COI* and *16S* rRNA, in the *16S* rRNA phylogenetic tree, we grouped RS-06 and RS-07 as *I. lineatus* and *I. edentulus*, respectively. Furthermore, RS-9 and RS-10 formed a monophyletic clade (grey color) with the *B. caudolineata* clade. The *16S* rRNA tree also revealed that each *B. caudolineata* sample from GenBank formed two distinct clades, with *B. caudolineata* (KX301918) genetically closer to RS-9 and RS-10, and *B. caudolineata* (KX301917) more closely related to RS-8. These clades were supported by high bootstrap values (NJ and ML: 97-100%) and posterior probability (1.00%).

The intrapopulation genetic variation was detected in three species namely *E. vermiculatus*, *I. lineatus*, and *B. caudolineata*. In *E. vermiculatus* (RS1 to RS5), 582 bp of *16S* rRNA sequences revealed two haplotypes and one variable site in position site of 478. That position site was transition pairs. No gap/indel was detected in five samples of *E. vermiculatus*. The nucleotide diversity and haplotype diversity were 0.00069±0.00041 and 0.400±0.237 respectively. In *I. lineatus* (RS-6 and RS-13), 587 bp of *16S* rRNA sequences showed two haplotypes with 50 variable sites and 12 gaps/missing data. The sites positions were 12, 19, 22, 23, 50, 54, 146, 157, 180, 181, 198, 204, 205, 239, 248, 249, 260, 264, 265, 266, 267, 269, 270,

272, 275, 288, 289, 290, 291, 300, 330, 332, 351, 352, 354, 357, 360, 361, 372, 377, 388, 389, 391, 410, 426, 427, 428, 444, 483, and 497. The transition pairs (si=29) were more frequent than transversion pairs (sv=21). The nucleotide diversity and haplotype diversity were 0.087±0,043; and 1,00±0,50 respectively. The genetic variation was also observed in *B. caudolineata* (RS-8) compared with RS-9 and RS-10. From 585 bp, two haplotypes were detected with 43 polymorphic sites, and five gaps or missing data. The site positions were 17, 19, 50, 51, 135, 159, 160, 163, 176, 180, 201, 203, 239, 241, 243, 244, 245, 248, 249, 261, 270, 275, 284, 288, 290, 291, 299, 333, 337, 346, 356, 357, 359, 369, 373, 376, 379, 381, 383, 392, 408, 421, and 438. The transition pairs (si=33) were more frequent than transversion pairs (sv=10). The nucleotide diversity and haplotype diversity were 0.0494±0,0233 and 0.667±0.099 respectively.

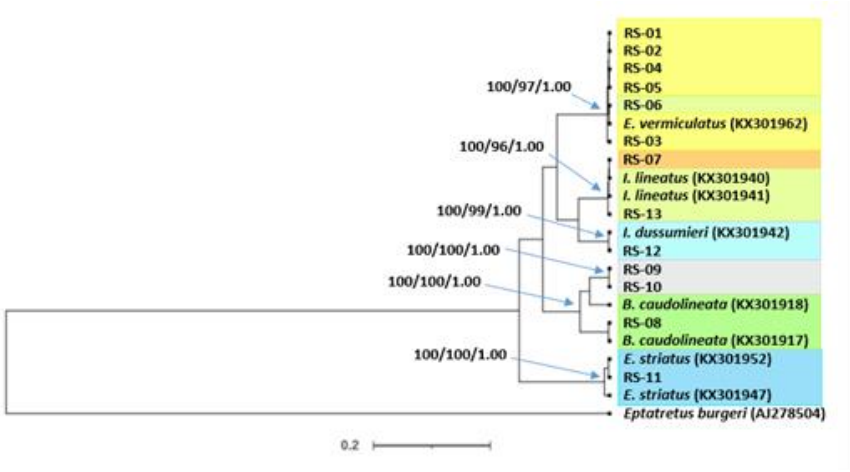

Figure 4. Bayesian phylogenetic tree inferred from mitochondrial *16S rRNA* sequences. The tree was produced from 10^7 generations using the GTR+G model. The number of each node represents posterior probabilities and the scale corresponds to substitutions/site

Phylogenetic analysis by combining COI and 16S rRNA

A total of thirteen Rockskipper sequences and an outgroup sequence, each formed through a combination of *COI* and *16S* rRNA, were aligned and yielded a clean sequence of 1195-1242 bp. Gaps/insertions/deletions were observed in all sequences. The phylogenetic tree showed identical topology in three different methods (NJ, ML, and BI), and only the Bayesian tree was displayed (Figure 5). The sequence combinations revealed the same topology as the *16S* rRNA phylogenetic tree (two clusters), and *E. striatus* was the basal taxon for all the Rockskipper species. In contrast to the *16S* rRNA tree, the combination tree of both genes revealed no species overlap, with all samples matched to their respective species (shown in colors). The species of the genera *Istiblennius* formed a monophyletic group with each other (*I. lineatus*, *I. edentulus*, and *I. dussumieri*), whereas the species of the genera *Entomacrodus* (*E.*

vermiculatus and *E. striatus*) formed polyphyletic group. RS-09 and RS-10 yielded similar results with the *COI* and *16S* trees, forming a clade with *B. caudolineata*.

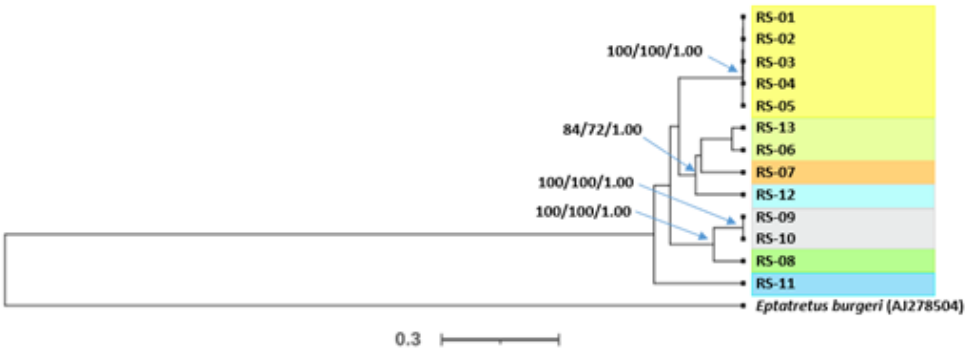

Figure 5. Bayesian phylogenetic tree inferred from combination of mitochondrial *COI* and *16S rRNA* sequences. The tree was produced from 10^7 generations using the GTR+G model. The number of each node represents posterior probabilities and the scale corresponds to substitutions/site

DISCUSSION

DNA barcoding based on genetic distance and phylogenetic tree approaches is an effective, efficient, and reliable method for species identification in a wide range of taxa (HEBERT *et al*., 2003a; HEBERT *et al*., 2003b; WARD *et al*., 2005; HAJIBABAEI *et al*., 2006). This study is the first study to employ *COI* and *16S* mtDNA sequences in Rockskipper species from Porok Beach, Gunungkidul Yogyakarta, to determine the taxonomic status and generate species-specific DNA barcodes for six species in the present study.

Using DNA barcoding of *COI* mtDNA, we discovered that the thirteen Rockskipper samples in this study, belong to six species (*E. vermiculatus*, *E. striatus*, *I. lineatus*, *I. dussumieri*, *I. edentulus*, and *B. caudolineata*) with a similarity more than 99% compared to the data on GenBank and BOLD, except for two unverified species (RS-9 and RS-10) with low similarity (93.43%) to *Blenniella caudolineata* from GenBank (KOENTJANA, 2016). In contrast to the *COI* findings, the *16S* rRNA analysis identified only five species. This phenomenon is attributed to the drawback of using the *16S* gene for species determination, as it exhibits a higher level of conservation compared to the *COI* gene. Due to its conservation, the *16S* gene is less effective in discriminating species when compared to the *COI* gene. The reduced variability in the *16S* gene may limit its ability to distinguish closely related species, making it less optimal for certain species delimitation. This limitation is particularly evident in Carps (MOHANTY *et al.*, 2015), Flatfish, and Gunard species (KOCHZIUS *et al*., 2010), in which *COI* has demonstrated greater effectiveness than *16S* rRNA for species identification. Thus, we exclusively used the *COI* gene for species verification since *COI* is more accurate and reliable in identifying species.

The six species of Rockskipper from Porok Beach were genetically distinct and divided into two groups based on the partial sequence information of *COI*, *16S* rRNA, and both gene combinations. The K2P intrapopulation genetic divergence of *COI* genes ranges from 0-11.81%.

The distance of 11.81% was between two unverified species (RS-09 and RS-10) when compared to *B. caudolineata* (RS-8) based on ZEMLAK *et al*. (2009) has exceeded the species threshold for fish (3,5%), indicating that RS-9 and RS-10 were separate species from RS-8 (*B. caudolineata*). The Rockskipper species had a relatively high degree of interspecies K2P nucleotide divergence in *COI* (17.35-23.61%) and *16S* (4.62-20.00%), showing the ability for both genes to sufficiently represent Rockskipper fish inter-relationships.

Three distinct Bayesian phylogenetic trees using *COI*, *16S*, and both gene combinations demonstrated that RS-9 and RS-10 form a monophyletic clade with maximum bootstrap support (100% in NJ/ML) and posterior probability (1.00 in BI), which further confirms that RS-9 and RS-10 are distinct species from *B. caudolineata*. Furthermore, both *COI* and *16S* rRNA trees showed that each *B. caudolineata* sample from GenBank formed two distinct clades: a *B. caudolineata* lineage from Guam (*COI*: KX301835; *16S*: KX301918) that was genetically closer to RS-9 and RS-10, and a *B. caudolineata* lineage from Taiwan (*COI*: KX301834; *16S*: KX30191) which was more closely related to RS-8. We assumed that *B. caudolineata* is a species complex that at least comprises two species. This species complex phenomenon was also observed in a study conducted by CHEN *et al*. (2014) using the *ND5* and *rag1* genes, which revealed that the Pacific Northwest mudskipper *Boleophthalmus pectinirostris* is a species complex consisting of two species: the East Asian population and the Malaysian population, with K2P genetic divergence ranging from 0.57% to 11.52%.

This study exhibited a T>C>A>G nucleotide composition pattern of the *COI* gene that was comparable to studies in other fish families (BINGPENG *et al*., 2018; WU *et al*., 2018; LINH *et al*., 2019; AJI and ARISURYANTI, 2021), whereas the *16S* nucleotide composition in this study consisted of two distinct patterns: A>C>T>G and A>C>G>T in which also consistent with studies in other fish families employing *16S* gene (LAKRA *et al*., 2010; BINEESH *et al*., 2015; WU *et al*., 2018; ARISURYANTI *et al*., 2020a; HABIB *et al*., 2021). The reported transitions and transversion ratios for COI and 16S genes in this study are comparable to several mtDNA teleost fish studies that have the number of transition pairs outnumbering the number of transversion pairs (LAKRA *et al*., 2010; WU *et al*., 2018; BINGPENG *et al*., 2018). All Rockskipper species in this study have high GC contents in the *COI* (46.52%) and *16S* rRNA (47.38%) regions. WARD *et al*. (2005) discovered that the total GC content of the whole mtDNA genome in fishes ranged from 38.4-43.2%, with *COI* ranging from 42.2-47.1%.

This study showed a high level of haplotype diversity (Hd $\geq$ 0.5) and nucleotide diversity ($\pi \geq 0.01$) between RS-9 and RS-10 samples compared to RS-8. In addition, a low level of haplotype diversity (Hd $\leq$ 0.5) and nucleotide diversity ($\pi \leq 0.01$) between RS-1 to RS-5 samples on both genes. According to GRANT and BOWEN (1998), a high haplotype and nucleotide diversity is caused by a large stable population with an extensive evolutionary history or secondary interaction between several lineages. In contrast, the low level of haplotype and nucleotide diversity formation is due to a recent population bottleneck or founder effect by a single or several mtDNA lineages.

In this study, *COI* and *16S* rRNA genes demonstrated their ability to discriminate species and revealed genetic variation among Rockskipper samples. However, our findings suggest that the *COI* gene is a more accurate and reliable marker for identifying Rockskipper species from Porok Beach, Gunungkidul, Special Region Yogyakarta. This finding provide data

to assist Rockskipper species conservation management programs in Indonesia. Additionally, the genetic data in this study can be utilized to organize and enhance the *COI* and 16S *rRNA* library of Rockskipper fish in Indonesia.

CONCLUSION

We successfully identified thirteen Rockskipper samples from Porok Beach that belong to six species (*E. vermiculatus*, *E. striatus*, *I. lineatus*, *I. dussumieri*, *I. edentulus*, and *B. caudolineata*) with more than 99% similarity. In contrast, the *16S* analysis identified five species. A Bayesian phylogenetic tree demonstrated that six species of Rockskipper from Porok Beach are genetically distinct and separated into two groups based on partial sequence information from *COI*, *16S* rRNA, and both gene combinations. We also found that RS-9 and RS-10 form a monophyletic group with *B. caudolineata* with maximum bootstrap support (100% in NJ/ML) and posterior probability (1.00 in BI). We hypothesized that *B. caudolineata* is a species complex with at least two lineages: one lineage is genetically closer to RS-8 and another is more closely related to RS-9 and RS-10. Both the *COI* and *16S* rRNA genes were capable of delineating species and revealing genetic variation among Rockskipper samples in this study. However, our findings demonstrated that the *COI* gene is a more accurate and reliable marker for identifying Rockskipper species from Porok Beach, Gunungkidul, Yogyakarta.

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MOLEKULARNA IDENTIFIKACIJA VRSTE *ROCKSKIPPER*-A (RIBE: BLENNIIDAE) SA PLAŽE POROK (JOGJAKARTA, INDONEZIJA) ZASNOVANE NA 16S rRNA i *COI* GENIMA

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IZVOD

Rockskipper pripada familiji Blenniidae (Combtooth blenni) bogatoj vrstama i poznat je po prisustvu kriptičnih i kompleksa vrsta. Identifikovanje kriptičnih vrsta zasnovano isključivo na morfološkim karakteristikama predstavlja izazov zbog njihovih sličnih morfoloških karakteristika. Kao rezultat toga, molekularne genetičke tehnike zasnovane na dva parcijalna gena mtDNK, *COI* i *16S* rRNA, korišćene su za preciznu identifikaciju riba. Ova studija je imala za cilj da proceni efikasnost *COI* i *16S* rRNA gena za identifikaciju ribe *Rockskipper* i istraži genetski odnos između vrsta *Rockskippera* sa plaže Porok. Rezultat je otkrio da je trinaest uzoraka *Rockskippera* sa plaže Porok koji pripadaju vrstama *E. vermiculatus, E. striatus, I. lineatus, I. dussumieri, I.dentulus i B. caudolineata* sa više od 99% sličnosti. Nasuprot tome, *16S* analiza je identifikovala pet vrsta. Bajesovsko filogenetsko stablo pokazalo je da je šest vrsta *Rockskippera* sa plaže Porok genetski različito i podeljeno u dva klastera. Takođe smo otkrili da dva uzorka (RS-9 i RS-10) formiraju monofiletsku grupu sa *B. caudolineata* sa maksimalnim *bootstrap-om* (NJ i ML: 100%) i posteriornom verovatnoćom (1,00). Pretpostavili smo da je *B. caudolineata* kompleks vrsta sa najmanje dve loze: jedna je bila genetski bliža RS-8, a druga je bila bliža RS-9 i RS-10. Utvrđeno je da su i *COI* i *16S* rRNA geni sposobni da razgraniče vrste i otkriju genetske varijacije među uzorcima *Rockskippera* u ovoj studiji. Međutim, naši nalazi su pokazali da je *COI* gen tačniji i pouzdaniji marker za identifikaciju vrsta *Rockskippera* sa plaže Porok, Gunungkidul, Jogjakarta, Indonezija.

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