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Determination of Grouper Species of Subfamily Epinephelinae from Raja Ampat (West Papua) Region Using CO1 Gene Sequence

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Abstract

The Raja Ampat Islands, located near the heart of the “Coral Triangle” on Bird's Head Peninsula, West Papua, Indonesia are well known for its outstanding biological diversity and stunning marine and terrestrial habitats. Groupers (family Serranidae) has included as part of the five largest families associated with coral reefs on the Bird’s Head region. The grouper identification was generally made based on color patterns and morphological characters, but often, these characters show intraspecific variations or differences in color patterns between juvenile and adult individuals. This study aims to confirm the type of grouper species obtained from the sport fishing activities around Raja Ampat Island. Species confirmation from the subfamily of Epinephelinae determined by analyzing the number of differences of nucleotides and genetic distance on the sequence of the CO1 gene (Cytochrome oxidase subunit 1). A total of eight fish samples were successfully sequenced and aligned. Those samples consist of eight species belonging to the three genera, namely Anyperodon, Epinephelus, and Cephalopolis.

Key Words: Epinephelinae, CO1, sports fishing, genetic distance, grouper, nucleotide

Introduction

The Raja Ampat Islands, located near the heart of the “Coral Triangle” on Bird's Head Peninsula, West Papua, Indonesia are well known for its outstanding biological diversity and stunning marine and terrestrial habitats. The Raja Ampat Island composed of four main islands (Waigeo, Batanta, Salawati, and Misool) and hundreds of smaller islands. The archipelago has one of the highest diversity of the world’s coral reef fish faunas, which consisted of at least 1074 species. Groupers (family Serranidae), including into the five largest families associated with coral reefs in the Bird’s Head region (McKenna *et al*. 2002).

The Serranid are a large family of fishes belonging to the order Perciformes. The Serranid Subfamily Epinephelinae commonly known as groupers, rockcods, hinds, and sea basses, comprises about 159 species of marine fishes in 15 genera. These commercially essential fishes are bottom-associated, which found in tropical and subtropical waters. Most species occupy coral reefs, but some inhabit estuaries or on rocky reefs (Heemstra and Randall 1993). Grouper has potential economic value in fisheries, many species such as *Epinephelus coioides, E. malabaricus, E. tauvina, E.marginatus*, *Cephalopholis boenak* known as consumption fish while the others also used as ornamental fish (Hemstra and Randal 1993; Asensio *et al*. 2009; Noitokr *et al*. 2013). Color patterns and morphological characters generally used for the grouper identification, but overlapping meristic counts and changes in a color pattern during life stages contributes to misidentification in grouper species (Hemstra & Randall 1993, Craig *et al*. 2001, Alcantara & Yambot 2014). It causes any kind fishes of this subfamily member caught in the field are often summed up as groupers (Ariyanti 2015).

A partial sequence of the mitochondrial cytochrome oxidase c subunit 1 (CO1) gene commonly used as a barcode with the size is about 650 bp. CO1 gene has widely used in several animal taxa such as insects, birds, and fish (Hebert *et al*. 2007). This gene has also been used in rapid analyses for commercial purposes, especially for the confirmation of fish species (Ward *et al*. 2005; Barber and Boyce 2006; Wong and Hanner 2008; Sachithanandam *et al*. 2012). This present work applied the partial CO1 as a molecular marker to delimitate the type of grouper species that obtained from sport fishing activities in around Raja Ampat Island.

Materials and Method

Fish species belonging to family Serranidae were collected from several sampling points in Raja Ampat areas specifically around Salawati island (Fig.1). Phenotypic characterization was analyzed using FAO species catalog of groupers of the world. Tissue samples that were used as the source of DNA are part of the dorsal muscle tissue. All tissue specimen was stored within 95% alcohol for the molecular genetic study.

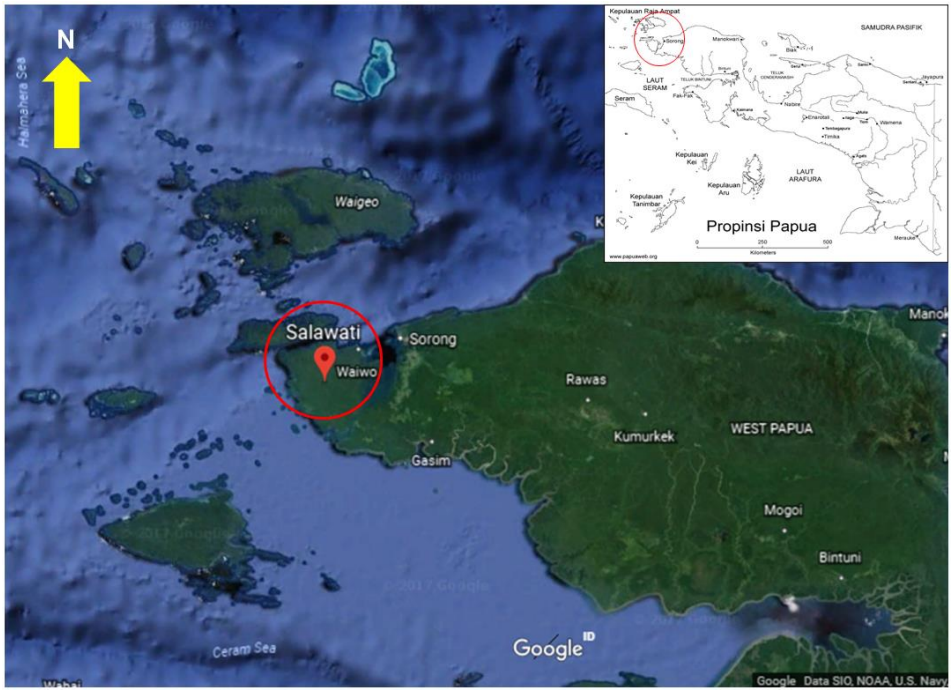

Figure 1. Location of Salawati Island in Bird's Head Peninsula, West Papua, Indonesia.

DNA Extraction and PCR Reaction

Total DNA was extracted from 0.30 g of ethanol-preserved tissue muscle using DNA Extraction Kit for animal tissue (Geneaid) by following the manufacturer's protocol. Approximately 650-655 bp were amplified from the 5′ region of the COI gene using combinations of the fish-specific primers AF282 and AF283 which is designed as a degenerate primer for fish were described in Ward *et al*. (2005).

The 50 µL PCR mixes included 25 µL 2X GoGreen GoTaq DNA polymerase mix (0.05 U/µL, 3mM Mg^{2+}, 0.4 mM each dNTP), 1 µL of each primer (1^{st} BASE, Singapore), 2 µL of DNA template and 21 µL of Nuclease-Free Water. The thermal regime consisted of an initial step of 2 min at 94 °C followed by 36 cycles of 0.5 min at 94 °C, 0.5 min at 56-57 °C, and 1 min at 72 °C, followed in turn by 7 min at 72 °C and then held at 4.0 °C in an Applied Biosystems (Foster City, CA, USA) Esco™ thermocycler.

Visualization

The amplicon was performed using a 6% polyacrylamide gel that runs at a voltage of 200 volts for 40 minutes. Afterward, proceed with silver staining.

Data Analysis

All amplicon were sequenced through company service sequencing following the manufacturer's protocol. DNA sequences were proofread, aligned, and edited using BioEdit (Hall 1999). Multiple alignments were done using Clustal W in MEGA 6 (Tamura *et al*. 2013). Sequence divergence was estimated using the Kimura two Parameters model of base substitution (Kimura, 1980). Phylogenetic tree reconstruction was done using the distance-based method, Neighbor-joining (NJ), and Maximum Likelihood (ML) as comparative trees base on CO1 region with nodes frequencies were calculated based on 1000 bootstrap replicates. Several sequences of grouper species used in GenBank (accession number in the figure) to root the tree for comparative purposes.

Result and Discussion

Each sequence in this work has aligned with an accuracy of homologous analysis result sequences from the GenBank (BLAST) about 98-100%. Those samples confirmed as *Epinephelus coeruleopunctatus* (RJ1), *E. ongus* (RJ5), *E. fasciatus* (RJ6), *E. melanostigma* (RJ7), *E. quoyanus* (RJ9), *E. coioides* (RJ4), *Anyperodon leucogramicus* (RJ3), *and Cephalopholis miniata* (RJ11). The partial CO1 sequences were 520 nucleotides (nt) with 188 nt variable sites, 332 nt conserved sites, and 169 nt parsimony informative sites. Based on the partial CO1 and using *Haemulon scuderii* as an outgroup, a molecular phylogenetic tree constructed by the Neighbor-Joining (NJ) method (Kimura 2-parameter). The values of the bootstrap confidence level of nodes indicated above the branch. Fig. 2 shows that all GenBank sequences and sequences of subfamily Epinephelinae acquired in this study. NJ tree exhibited 2 clades that separate 3 genera (including *Anyperodon*).

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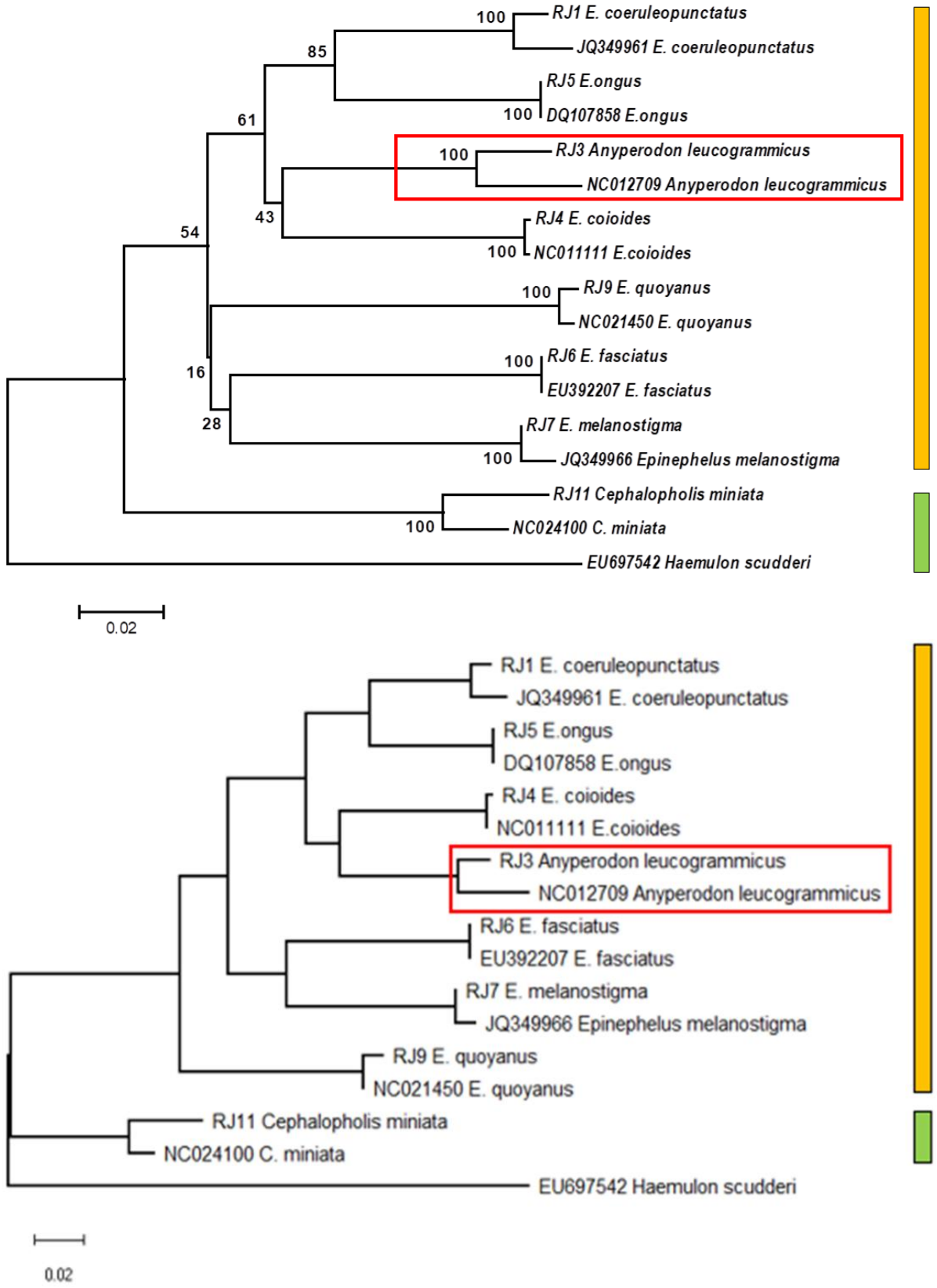

Figure 2. Neighbor-joining and Maximum Likelihood (ML) as comparative trees based on the mtDNA CO1 nucleotide sequences of groupers samples. Numbers at nodes are bootstrap values on 1000 replicates.

Table 1. Number of differences of nucleotides (below the diagonal) and genetic distances of CO1 (above the diagonal) in present work

No	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	RJ1 (*E. coeruleopunctatus*)		0.139	0.127	0.099	0.175	0.153	0.165	0.170	0.151	0.175	0.099	0.162	0.157	0.125	0.022	0.183	0.262
2	RJ3 (*Anyperodon leucogrammicus)*	64		0.117	0.135	0.159	0.169	0.167	0.205	0.042	0.159	0.135	0.179	0.164	0.119	0.148	0.184	0.245
3	RJ4 (*E. coioides*)	59	55		0.119	0.146	0.152	0.167	0.217	0.131	0.146	0.119	0.161	0.165	0.002	0.134	0.201	0.265
4	RJ5 (*E.ongus*)	47	63	56		0.174	0.127	0.175	0.204	0.143	0.174	0.000	0.136	0.172	0.121	0.105	0.204	0.265
5	RJ6 (*E. fasciatus*)	79	73	68	79		0.142	0.159	0.212	0.164	0.000	0.174	0.151	0.162	0.144	0.174	0.191	0.254
6	RJ7 (*E. melanostigma*)	70	77	70	60	66		0.159	0.206	0.169	0.142	0.127	0.008	0.167	0.150	0.159	0.209	0.236
7	RJ9 (*E. quoyanus*)	75	76	76	79	73	72		0.181	0.169	0.159	0.175	0.168	0.008	0.165	0.172	0.168	0.301
8	RJ11 (*Cephalopholis miniata*)	78	91	96	91	94	91	82		0.208	0.212	0.204	0.216	0.178	0.215	0.183	0.040	0.266
9	NC012709 *Anyperodon leucogrammicus*	69	21	61	66	75	77	77	92		0.164	0.143	0.179	0.167	0.134	0.156	0.184	0.262
10	EU392207 *E. fasciatus*	79	73	68	79	0	66	73	94	75		0.174	0.151	0.162	0.144	0.174	0.191	0.254
11	DQ107858 *E.ongus*	47	63	56	0	79	60	79	91	66	79		0.136	0.172	0.121	0.105	0.204	0.265
12	JQ349966 *Epinephelus melanostigma*	74	81	74	64	70	4	76	95	81	70	64		0.176	0.159	0.159	0.219	0.247
13	NC021450 *E. quoyanus*	72	75	75	78	74	75	4	81	76	74	78	79		0.162	0.164	0.165	0.298
14	NC011111 *E.coioides*	58	56	1	57	67	69	75	95	62	67	57	73	74		0.131	0.198	0.267
15	JQ349961 *E. coeruleopunctatus*	11	68	62	50	79	73	78	83	71	79	50	73	75	61		0.181	0.274
16	NC024100 *C. miniata*	83	83	90	91	86	92	77	20	83	86	91	96	76	89	82		0.251
17	EU697542_*Haemulon_scudderi*	114	108	115	115	111	105	127	115	114	111	115	109	126	116	118	110	

Based on table 1, the smallest number of differences nucleotides was 47 nt, between RJ1 and RJ5. Then the biggest is 96 nt between RJ4 and RJ11. The differences number of nucleotides seem like appropriate with genetic distances. Minimum genetic distances value in CO1 among all *Epinephelus* genera was 0.099 between RJ1 (*E. coeruleopunctatus)* and RJ5 (*E. ongus*). The maximum genetic distances value is RJ5 and RJ9, RJ1 and RJ6 with genetic distance 0.175 respectively. The maximum pairwise nucleotide divergence value between RJ4 and RJ11 is 0.217.

Neighbor-Joining tree exhibited 2 clades that separate 3 genera (including *Anyperodon*). The phylogenetic analysis showed the lowest genetic distance between *Epinephelus* and *Anyperodon* (Table 1). It shows in Fig. 2 that *Anyperodon leucogrammicus* was grouped within *Epinephelus* genera so that the cluster become not monophyletic. This fact confirms the paraphyletic status of the *Epinephelus* (Craig *et al.* 2001; Zhu *et al*. 2008; You *et al*. 2013). Current classification, *Anyperodon* is distinctive monotypic genus is probably most closely related to *Epinephelus*, with which it shares XI dorsal-fin spines and the absence of trisegmental pterygiophore, but it differs from *Epinephelus* (and all other groupers) in its missing teeth on the palatines. *Anyperodon* is also unique among groupers in its elongate groupers, but none of these specimens are looking as compressed as *Anyperodon* (Hemstra & Randall 1993).

The position *Epinephelus* and *Cephalopholis* in the phylogenetic tree also similar with Craig *et al*. (2001), using 16S gene and then confirmed by Craig and Hasting (2007) that support the valid genus of the *Cephalopholis* separate from *Epinephelus*. *Cephalopholis* is more primitive than genus *Epinephelus. Cephalopholis* is one of the wealthiest genera (besides *Mycteroperca* and *Epinephelus*) which has various species. The NJ tree shows the *C. miniata* become sister clades of *Epinephelus* clades. The position of *Epinephelus* at the top of the phylogenetic tree indicating that is the most recently diverged species, which is in concordant with the fact that it is also the most advanced genus in Epinephelinae (Craig *et al*. 2001; Craig and Hasting 2007, Ding *et al*. 2006).

E. quoyanus is one of 9 shallow-water coral reef species that have a rounded caudal fin and close-set dark brown spots with the pale interspaces forming a network on the body. These reticulated groupers have been much confused in the literature, and many museum specimens have misidentified with the other species such as *E. bilobatus, E. faveatus, E. hexagonatus, E. macrospilos, E. maculatus, E. melanostigma, E. merra, and E. spilotoceps* (Hemstra and Randall 1993).

The difficulties of groupers species identification and confirmation based on morphological characters could be resolved by identification using partial CO1. Also, the genetic relationship of the groupers successfully reconstructed through the phylogenetic analysis. Partial cytochrome oxidase c subunit 1 (CO1) gene successfully delimitate the type of grouper species and reveal the diversity of groupers species that obtained from the sport fishing activities in around Raja Ampat Island.

Conclusion

Species confirmation and determination of subfamily Epinephelinae did by analyzing the differences between nucleotide and genetic distance on the segment of the CO1 gene (Cytochrome oxidase subunit 1). A total of eight fish samples were successfully sequenced and aligned with data from the GenBank. Those samples consist of eight species belonging to three genera, namely *Anyperodon*, *Epinephelus*, and *Cephalopolis.* This work exhibit the exceptional diversity of groupers species from Raja Ampat, as being shown from the samples acquired from sport fishing activities.

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