

## Status of *Epinephelus Fuscoguttatus* and *Epinephelus Hexagonatus* Inferred from Mitochondrial DNA Sequence (Cytochrome b): Evidence for Genetic Similarity

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**Abstract**—The degree of conservation of sequence using the cytochrome b gene from mitochondrial DNA is sufficient to discriminate between closely related species. A partial DNA sequence of cytochrome b gene was used to investigate the molecular relationship between *Epinephelus hexagonatus* and *Epinephelus fuscoguttatus*. Polymerase Chain Reaction (PCR) and sequence analysis were used to determine the genetic variation in approximately 450-nucleotides region of the mitochondrial cytochrome b gene in 160 fresh tissue samples. Phylogenetic trees were constructed with ME method, NJ method and MP method with bootstrapping using arithmetic average method. There are no differences or only small differences were detected between these two taxa. The results showed that *Epinephelus fuscoguttatus* clustered with *Epinephelus hexagonatus*, based on the phylogenetic trees *Epinephelus hexagonatus* and *Epinephelus fuscoguttatus* consistently clustered together with following the suggestion referring on their morphological data, that they may be closely related, or possibly placed within the same species.

**Keywords**—Phylogenetic tree; cytochrome b; mitochondrial DNA; species identification; *Epinephelus fuscoguttatus*; *Epinephelus hexagonatus*.

### I. INTRODUCTION

Throughout the history, the most important marine animals used in aquaculture production have been groupers (*Epinephelinae spp.*, *Serranidae*). Because of their higher market price compared to other fish, groupers are considered to be an exclusive and highly priced food. Groupers are difficult to find and hard to catch because they can only be found in the deep sea [1]. They are fast-growing, disease-resistant and suitable for culture, indicating that they possess excellent biological characteristics. Grouper farming appeared to have great promise, and coastal cage culture has potential for continued development. However, growth of the grouper culture industry has been hindered by an unreliable and limited supply of seed of the *Epinephelus* species. Controlled reproduction from simultaneously available mature broodstock of both sexes is required for the mass production of grouper fry in hatcheries, and this will certainly be the key to continued growth of the industry [2]. Across hundreds of millions years of evolution, major changes in regulatory genes have produced important evolutionary changes in body plan and morphology, which is why the molecular marker approach has been essential for

finding and tracking ancient and new species and especially for phylogenetic tree development.

Reference [3] studied of 28 grouper species within six genera of the subfamily *Epinephelinae* and used cytochrome b to derive the phylogenetic relationships of groupers in the China Seas. They concluded that the genus *Plectropomus* (*Serranidae, Perciformes*), which diverged early, is the most primitive group in the subfamily *Epinephelinae*; the genus *Variola* (*serranidae, Perciformes*) is more closely related to *Cephalopholis* (*Serranidae, Perciformes*) than to the other four genera; *Cephalopholis* is a monophyletic genus and more primitive than *Epinephelus*; *Promicrops lanceolatus* (*Serranidae, Perciformes*) and *Cromileptes altivelis* (*Chromileptes, Serranidae*) should be included in the genus *Epinephelus*; and there exist two sister groups in *Epinephelus*. The phylogenetic relationships among the fishes in the *perciform* tribe *Epinephelinae* (*Epinephelus, Serranidae*) have long been poorly understood in large part because of the numerous taxa that must be considered and the large, circumtropical distribution of the group. Knowledge of relationships within the *Serranidae* has been equally tenuous [4].

Cytochrome b sequences are commonly used to evaluate vertebrate phylogenies. Investigations among closely related species are possible because cytochrome b marker evolve rapidly. Cytochrome b sequences also contain slowly evolving sites that facilitate the investigation of deeper relationships and can improve species identification. Between closely related taxa and distant taxa, cytochrome b sequence diverges faster than nuclear DNA sequence, and the sequence divergence continues to increase in nuclear DNA [5].

Recently a few questions were raised on the *Epinephelus* species on their morphological similarities and the species had extensive phonetic similarities, suggesting that some species in *Epinephelinae sp.* might belong in a same species and group [5];[6]. However, in a previous molecular pylogenetic study focusing on serranid fish genus *Epinephelus* [7] *Mycteroperca* [5] and *Plectropomus* [6] showed no or only provided general data on *Epinephelus hexagonatus* in their studies.

In our study, we carried out cytochrome b (cyt b) analysis as molecular marker to investigate the molecular relationship between *Epinephelus hexagonatus* and *Epinephelus fuscoguttatus*. Analysis from BLAST database of partial

sequences of mtDNA cyt b of *Epinephelus fuscoguttatus* showed 99% similarity to the *Epinephelus hexagonatus* and that this two species are closely related and the level of differentiation on morphology between them is very low. This database has trigger a question, is these two species is a same species but have different name? Therefore, this study will carry out experiments that work on identifications and differentiations between those two species to answer some of the question that had arose time to time.

## II. MATERIALS AND METHODS

### A. Collection and Preservation

A piece of caudal fin area (20-50 mg) was individually collected from a live single *Epinephelus fuscoguttatus*. The specimens were collected from the Fisheries Research Institute, Terengganu Malaysia. A total of 160 individual initially identified as *Epinephelus fuscoguttatus* were subjected to the study. The specimens preserved in 95% ethanol at ambient temperature upon sampling and kept at -20°C under laboratory conditions. The specimens were homogenized in chilled nuclei lysis solution and kept at -20°C prior to be used.

### B. DNA Extraction

DNA extraction was based on the commercial kit from Promega with some modification according to the tissues (blood, fin or liver). DNA quantity was determined against 1kb molecular marker standard by electrophoresis in a 1% of agarose gel.

### C. PCR Amplification

Polymerase Chain Reaction (PCR) was used to amplify approximately 490bp fragment of the mtDNA cyt b gene. In both genes double-stranded DNA was PCR synthesized in 25 µl reactions (5 µl 5X Green GoTaq® Flexi Buffer, 2 µl 25 mM MgCl<sub>2</sub>, 1 µl 10 mM of each primer, 0.5 µl dNTP 0.2 mM/dinucleotide, 1 µl template DNA, and 0.5 µl (5u) GoTaq® DNA Polymerase (Promega Corporation©).

30 cycles of the following step procedure were performed using Eppendorf Master Cycler, the thermo cycler conditions were: preheat at 94 °C for 120 s (seconds), denaturation 94 °C for 5 min, annealing 45 °C for 2 min, extension 72 °C for 1 min, repeated for 1 cycle, followed by 29 cycles at 94 °C for 5 min, 45 °C for 2 min, 72 °C for 1 min and hold in 4°C. A set of primer used was: CytbFR 5'-CGCCTGTTTATCAAAAACAT-3' and CytbRV 5'-CCGGTCTGAACTCAGATCACGT-3' [8].

### D. Sequencing

PCR amplification products were purified on a 1% of agarose gel stained with Ethidium bromide (EtBr). Products of interest were identified using HindIII DNA ladder as a reference marker. PCR fragments were separated from the gel using a Wizard-prep PCR purification kit (Promega Corporation©). Automated fluorescent dye-deoxy sequencing of both strands was carried out using an ABI Prism 3100 DNA-Sequencer. Sequences from both strands of all individuals from each species were assembled into a

single consensus sequence using the assembly editor option in the Biology Workbench (ver. 3.2) and the tools that were used known as Nucleic Tools which combine general sequence analysis applications, with applications that are specifically for analyzing DNA or RNA and the second tool that were used known as Alignment Tools which allow to view and analyze aligned sequences.

Consensuses of the sequences were aligned using the alignment program Clustal W with default settings [9]. Indels were coded as a single character so as not to place undue character weight on a single mutational event leading to the insertion or deletion of multiple bases and was excluded from the analysis due to the ambiguity of the aligned characters.

### E. Phylogenetic Analysis

*Epinephelus bruneus* (accession No: NC013820) was used as an out-group when constructing the phylogenetic tree. Since variation within the nuclear loci was low, relationships between sequences for each locus were determine and the data set was analyzed with maximum parsimony (MP), molecular evolution (ME), and neighbor-joining (NJ) using the program MEGA 4.0 [10]. Sites with missing data were removed and the mitochondrial region sequences were used to test models of evolution. The p-distance model of evolution was used and employed to MP, ME and NJ tree the nucleotide sequences and relations were analyzed MP, ME and NJ as implemented in MEGA 4.0 [10]. Support for nodes was estimated using the bootstrap technique with 100 replicates. All new sequences were deposited in Genbank as in Table 1.

TABLE I. LIST OF TAXA SEQUENCED AND THEIR GENBANK ACCESSION NUMBERS

| Label of Fish Samples               | GenBank Accession No. |
|-------------------------------------|-----------------------|
| <i>Epinephelus hexagonatus</i> 3C11 | GU591708              |
| <i>Epinephelus hexagonatus</i> 3C27 | GU591711              |
| <i>Epinephelus hexagonatus</i> 4C7  | GU591702              |
| <i>Epinephelus hexagonatus</i> 4C10 | GU591703              |
| <i>Epinephelus hexagonatus</i> 4C13 | GU591704              |
| <i>Epinephelus hexagonatus</i> 4C17 | GU591705              |
| <i>Epinephelus hexagonatus</i> 5C18 | GU591718              |
| <i>Epinephelus hexagonatus</i> 6C6  | GU591720              |

## III. RESULTS

The cytochrome b gene was partially sequenced for all individuals examined. The numbers of base substitutions per site from analysis between sequences are based on the pairwise analyses of 160 sequences. The evolutionary history was inferred using the Neighbour-Joining (NJ), Minimum Evolution (ME), and Maximum Parsimony (MP).

For the Maximum Parsimony (MP) method, this tree out of 2412 most parsimonious trees (length = 179) is shown. The consistency index is (0.666667), the retention index is (0.954545), and the composite index is 0.922550 (0.636364) for all sites and parsimony-informative sites (in parentheses). The MP tree was obtained using the Close-Neighbor-Interchange algorithm [12] with search level 3 [11]; [12] in

which the initial trees were obtained with the random addition of sequences (10 replicates). While the Minimum Evolution (ME) method, The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 3.

Last but not least, the Neighbor-joining algorithm [13] was used to generate the initial tree. The evolutionary history that inferred using the Neighbor-Joining method showed the optimal tree (Fig. 1) with the sum of branch length = 0.95095399. The bootstrap consensus for Maximum Parsimony (MP), Minimum Evolution (ME) and Neighbor-Joining (NJ) tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed [11].

These trees branches corresponding to partitions reproduced in less than 60% bootstrap replicates are collapsed and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches [11]. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

There were a total of 340 positions in the final dataset for every set of the trees and out of which 8 were parsimony informative for the MP tree. These Phylogenetic analyses were conducted in MEGA4 [10].

#### IV. DISCUSSION

Mitochondrial DNA is a crucial marker allowing researchers to recognize and identify this *Serranidae* species for the many advantages that it offers. For evaluating genetic diversity and phylogeny, modern molecular biology has enabled comparisons between nucleotide and amino acid sequences of different populations. Many studies such as [14] successfully studied on *Osmeridae*, reference [15] on *Epinephelus quoyanus*, reference [16] on yellow grouper, reference [5] on *Epinephelus* and *Mycteroperca* species, reference [17] on *Cottidae*, and reference [18] on *Glyptosternoid* species have demonstrated that polymorphic loci or gene sequences are often conserved between closely related taxa. As a genetic marker for phylogenetic analyses, mitochondrial DNA has many advantages.

The cytochrome b (cyt b) gene has been sequenced more often than any other mitochondrial gene [19]. Partial nucleotide sequences of cytochrome b gene were aligned together and three types of phylogenetic trees were constructed using the Maximum Parsimony (MP), Molecular Evolution (ME) and Neighbour-Joining (NJ). The topologies of MP, ME and NJ (Fig. 1) trees were similar. The 160 individual of samples were clustered into 3 cluster groups with high bootstrap and consensus resampling support. Those 3 groups can be occurred with so many possibilities; some of the possibilities are variations on the tandem repeats [20], mutations (deletion/insertion/substitution) on the genes [21], genetic drifts as a result of small effective population sizes ecological diversity [22] and many more. These possibilities can also be used to describe the interference of *Epinephelus hexagonatus* into the cluster of *Epinephelus fuscoguttatus*.

Based on the figure of the phylogenetic tree of MP, ME and NJ (Fig. 1), the group cluster of the trees have mix

individuals of those two different species. This bring so many speculations and questions on the *Epinephelus hexagonatus*, are they belong in the same group to the species of *Epinephelus fuscoguttatus*? Or it happens because of the interference in their genetic makeup? Or probably had natural hybrid between those two species which cause it to carry some of the *Epinephelus fuscoguttatus* and *Epinephelus hexagonatus* genes? Or *Epinephelus hexagonatus* is misclassified as *Epinephelus fuscoguttatus*, which would therefore have the *Epinephelus hexagonatus* in the *Epinephelus fuscoguttatus* group and these has been reported by reference [23] that done a study on *Barbus* (*Cyprinidae*).

The answer maybe lies within the hatchery, ponds, or tanks that kept those fishes for broodstock management. Malaysia is striving for big commercialization of the aquaculture and fishery industry. However, there is no complete documentation [24] available on the broodstocks species and management. Most of the hatchery institutions bought their fry or fish larvae and the broodstock from local farms. Unfortunately, those farmers cannot identify any different species correctly just based on morphology itself and do not know how to handle the evolutionary aspects. As a consequence, the history, origin, size and sex ratio composition of the breeding stocks are frequently not optimally controlled. This bring so much concerned that repeated, uncritically advocacy has the potential to diminish recognition of the remaining gaps in thus knowledge and there, diminish support for continued research and development of an important management tool [25] raise expectations in the fishing community and in conservation circles that might prove unachievable; lead to neglect of other effective techniques for managing fishing effort; and result in expenditure of scarce financial and other resources in the creation and management of no-take reserves that are inappropriately sized or sited.

In an attempt to identify *Epinephelus fuscoguttatus* at the early stage of this study, its sequences were subjected to BLAST analysis with the available sequences in the Genbank. The results showed 98% - 99% similarity with *Epinephelus hexagonatus*. From the analysis, genetic distances were needed to be done to know exactly which group or species it belongs to.

#### V. CONCLUSION

Consequently, genetic monitoring of hybridization programs must be applied in a routine way, mainly to verify if trade and management of hybrids are correctly carried out in the fish breeding farm which can result in fertile hybrids and the variability of backcrossing. Furthermore, reports of these possible hybrids are not found, since *Epinephelus hexagonatus* have great morphologic similarity with the *Epinephelus fuscoguttatus* species, and genetic markers were not accessible in the identification of possible hybrids collected in the nature.

Therefore the correct identification could be very useful in developing broodstocks of *Epinephelus fuscoguttatus* which are known to be popular cuisines in Asia compared to the *Epinephelus hexagonatus* for profitable and sustainable

aquaculture of this species. Moreover, correct identification of grouper species might support grouper conservation programs.

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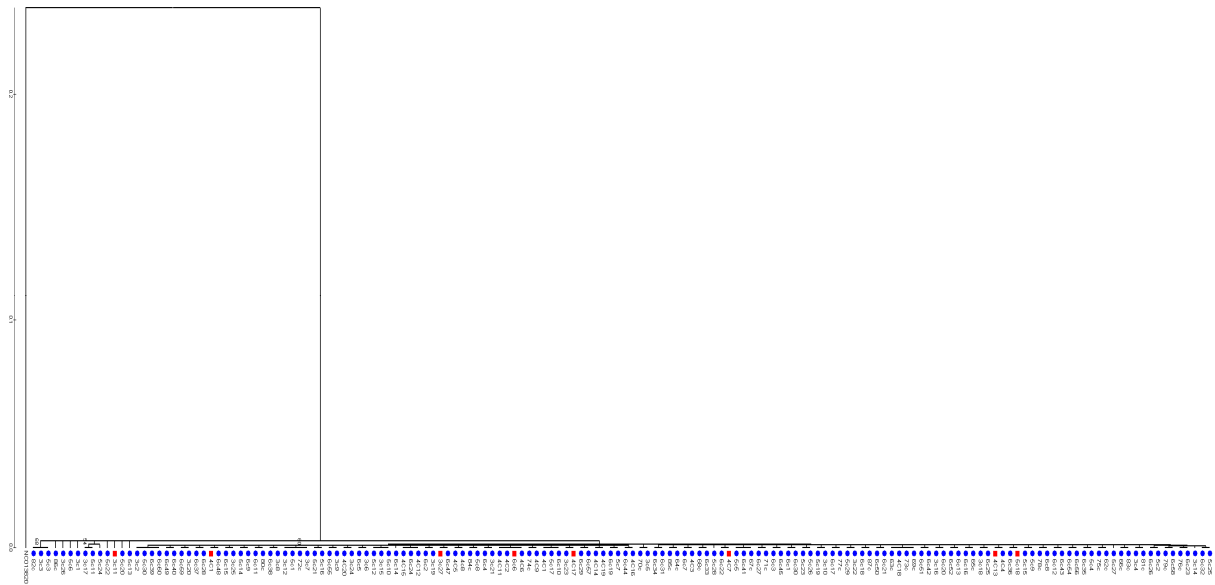


Figure 1. Evolutionary relationships of 160 taxa: blue represent *Epinephelus fuscoguttatus* and red represent *Epinephelus hexagonatus*.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.95095399 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 372 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4.0.