DNA barcoding and phylogenetic analysis of Malaysian groupers (Subfamily: Epinephelinae) using mitochondrial Cytochrome c oxidase I (COI) gene

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Abstract

The present study was carried out to examine the species identification and phylogenetic relationships of groupers in Malaysia using mitochondrial Cytochrome c Oxidase I (COI) gene, commonly known as barcoding gene. A total of 63 individuals comprising 10 species from three genera were collected from the coastal areas of Johor, Kelantan, Pahang, Perak, Selangor and Terengganu. All the individuals were morphologically identified and molecular works involved polymerase chain reaction (PCR) and sequencing of COI barcoding fragment (655 base pairs). Results from the BLAST search showed that 55 sequences could be assigned to 10 grouper species with high percentage identity index ($\geq$ 95% to 100%), while eight grouper individuals showed discrepancies in their taxonomic identification based on the morphology and the COI barcoding results. The histogram of distances showed that there was a clear-cut barcode gap present in the sequences indicating a clear separation between intraspecific and interspecific distances. The pairwise genetic distances showed lowest pairwise distance between *P. leopardus* and *P. maculatus* (4.4%), while the highest pairwise distance was between *E. bleekeri* and *P. maculatus* (23.5%), supporting their morphological and habitat similarities and differences. Phylogenetic analysis (Neighbor-Joining) showed the presence of two major clades (1) genus *Epinephelus* vs (2) genus *Plectropomus* and *Cephalopholis).* In conclusion, the present study has managed to show the accuracy of DNA barcoding method for species identification, and utilization of COI gene for phylogenetic study among groupers.

Introduction

Grouper or locally known as 'Ikan Kerapu' in Malaysia, is one of the commercialised fish that are often being caught by the fishermen along the coast of Malaysia. Grouper is a tropical marine fish, which usually inhabits coral reefs or sandy, silty or rocky areas in estuaries (Allen, 1999; Pierre *et al.*, 2007). Some grouper species are being widely cultured throughout the world to meet market demands. Their natural harvests are also of considerable economic value, especially for coastal fisheries in subtropical and tropical areas (Pierre *et al.*, 2007).

According to the International Union for Conservation of Nature (IUCN) Red List (2011), more than 90% of the coastal human population obtain their source of

food and income through fishing activities, and groupers are among the fish that are constantly being caught (Noikotr *et al*., 2013). This has resulted in reduced numbers of wild groupers nowadays due to uncontrolled exploitation of seed and adult stocks as well as the use of illegal collecting methods (Susanto *et al*., 2011). A few grouper species are already on the IUCN Red List, which include the Goliath grouper (*Epinephelus itaraja*) as a critically endangered, and Nassau grouper (*Epinephelus striatus*) as an endangered species.

The DNA barcoding method utilizing the Cytochrome c oxidase I (COI) gene fragment has proved to be an important method in resolving problems in taxonomy and species identification in various groups of organisms (Hebert *et al.,* 2003). Since groupers are usually identified using visible morphological, meristic and anatomical characters (Noikotr *et al*., 2013), they may lead to problem of misidentification due to overlapping of certain characters (Sachithanandam *et al*., 2011) and use of some taxonomic keys that requir high level of expertise as the keys might become confusing (Hebert *et al.,* 2003). Nevertheless, fish have become one of the easiest animal groups to generate DNA barcode data (Weigt *et al*., 2012). Thus, the objective of the present study was to genetically identify wild grouper species in Malaysia and to infer their phylogenetic relationships by using the mitochondrial COI barcoding gene.

Materials and Methods

Sample collections : A total of 63 individuals of grouper were collected from six sites throughout the Peninsula Malaysia (Fig. 1). All the samples were caught by fishermen and some were personally caught by a fishing rod. Morphological identification was done following keys provided by Mansor *et al.* (1998) and Atan *et al.* (2010). Fish samples (fin clipping, scale, muscle tissue or whole fish)

were preserved in 95% ethanol or kept on ice during field collection, and were subsequently stored at -20 °C prior to genetic analyses.

DNA extraction, polymerase chain reaction (PCR) and sequencing: DNA extraction of the fish tissues were done using Wizard® SV Genomic DNA Purification System (Promega). Extracted DNA was re-dissolved in 100μl of sterilised distilled water. The DNA quality and approximate yield were determined by a spectrophotometer or by electrophoresis in a 1% agarose gel containing GelRed™ Nucleic Acid Gel Stain (Biotium) solution at 90 V for 30 min. The isolated genomic DNA was used for genetic analysis.

A 655-base pair segment of the COI gene was amplified with oligonucleotide primers FishF1 (5'-TCAACCAACCACAAAGACATTGGCAC-3', forward) and FishR1(5'-TAGACTTCTGGGTGGCCAAAGAATCA-3', reverse) (Ward *et al.,* 2005). Approximately, 50-100 ng of template DNA was amplified in a 25 μl reaction mixture containing 50 mM 10x buffer, 25 mM MgCl$_2$, 40mM of dNTPs (Promega), 0.1 M of each primer, and 0.5 units of *Taq* DNA polymerase (Promega). The cycle parameters consist of 30 cycles of denaturation (at 95°C for 30 sec), annealing (at 52°C for 45 sec), and extension (at 72°C for 60 sec). The amplified products were visualized on 1% agarose gels containing GelRed™ Nucleic Acid Gel Stain (Biotium), run for approximately 60 min at 75 V, and photographed under UV light. A digested lambda DNA ladder (GeneRulerTM 100-bp DNA Ladder) was used as a standard size marker (Promega). The PCR products were further purified using a DNA purification kit (Promega) according to the manufacturer's instructions. All purified PCR products were directly sequenced by Genetic Analysers System (Applied Biosystems). The sequencing was carried out using only forward primer (FishF1). A sequencing reaction using reverse primer (FishR1) was subsequently carried out on

Table 1 : Species comparisons between identified species with their nearest species based on intraspecific and nearest neighbour distances. (Abbreviation: Sp.=Species, NN=Nearest Neighbour, N/A=Not Available/Singleton)

Species	Mean Intra-Sp	Max Intra-Sp	Nearest species	Distance to NN
C. boenak	1.27	3.92	C. formosa	9.45
C. formosa	N/A	N/A	C. boenak	9.45
E. areolatus	2.2	4.12	E. bleekeri	11.08
E. bleekeri	0.49	0.96	E. areolatus	11.08
E. coioides	0.29	0.48	E. fuscoguttatus	10.26
E. corallicola	0.48	0.48	E. fuscoguttatus	11.8
E. fuscoguttatus	0.16	0.16	E. coioides	10.26
E. heniochus	N/A	N/A	E. coioides	14.08
P. leopardus	N/A	N/A	P. maculatus	4.47
P. maculatus	N/A	N/A	P. leopardus	4.47

Table 2 : List of haplotypes sequences from each species with their accession number

Identified species	Gen bank accession numbers	Number of haplotypes
Cephalopholis boenak	KR863482 - KR863489	8
Cephalopholis formosa	KR863490	1
Epinephelus areolatus	KR863491 - KR863497	7
Epinephelus bleekeri	KR863498 - KR863503	6
Epinephelus coioides	KR863504 - KR863507	4
Epinephelus corallicola	KR863508 - KR863509	2
Epinephelus fuscoguttatus	KR863510 - KR863511	2
Epinephelus heniochus	KR863512	1
Plectropomus leopardus	KR863513	1
Plectropomus maculatus	KR863514	1
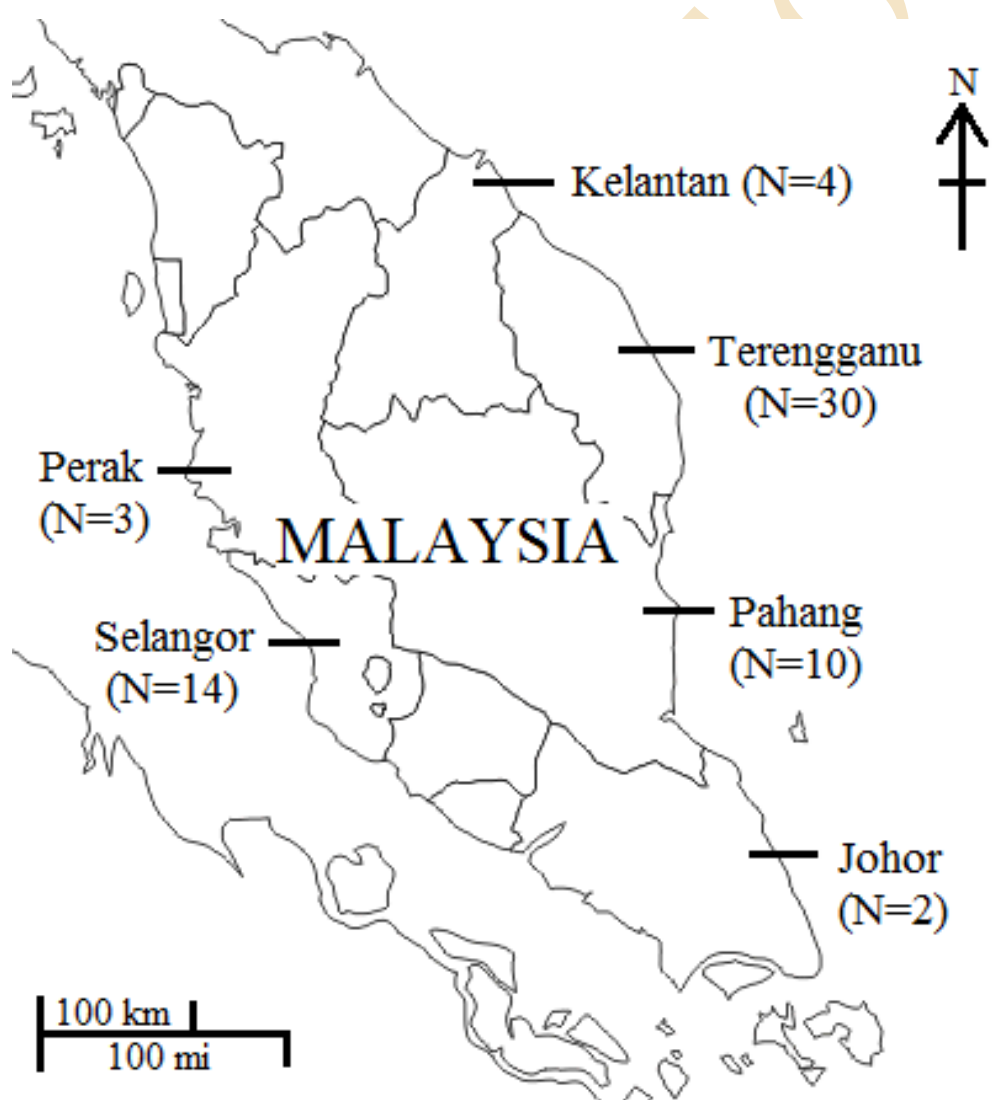
Fig. 1 : Areas covered during the study and total number of individual fish species caught in an area (Perak=3, Selangor=14, Johor=2, Pahang=10, Terengganu=30 and Kelantan=4)
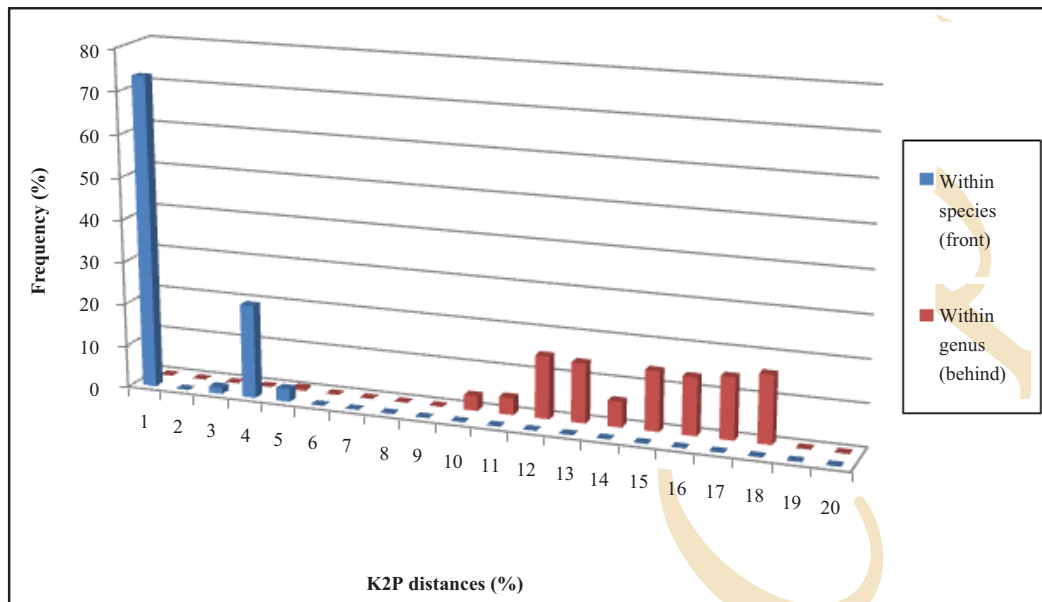
Fig. 2 : Histogram of normalised divergence for species against the genus divergence prior to Kimura 2 Parameter distances
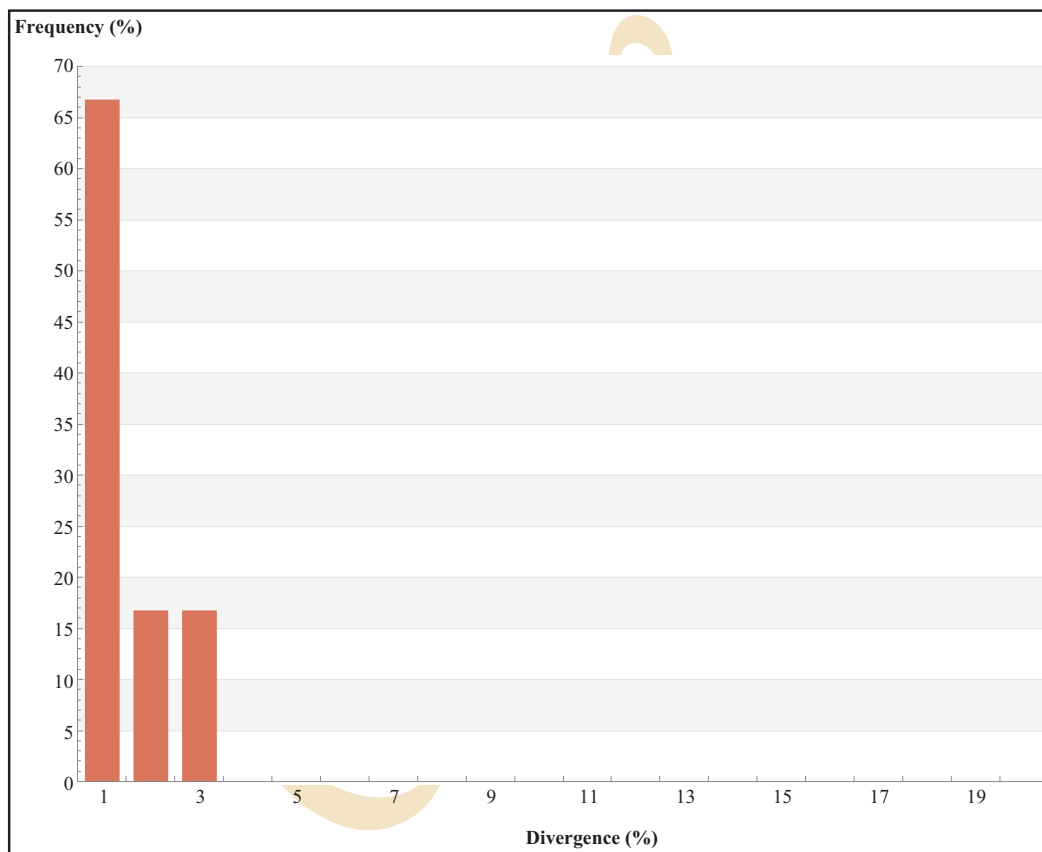

Fig. 3 : The histogram plots the mean intraspecific distances for each species
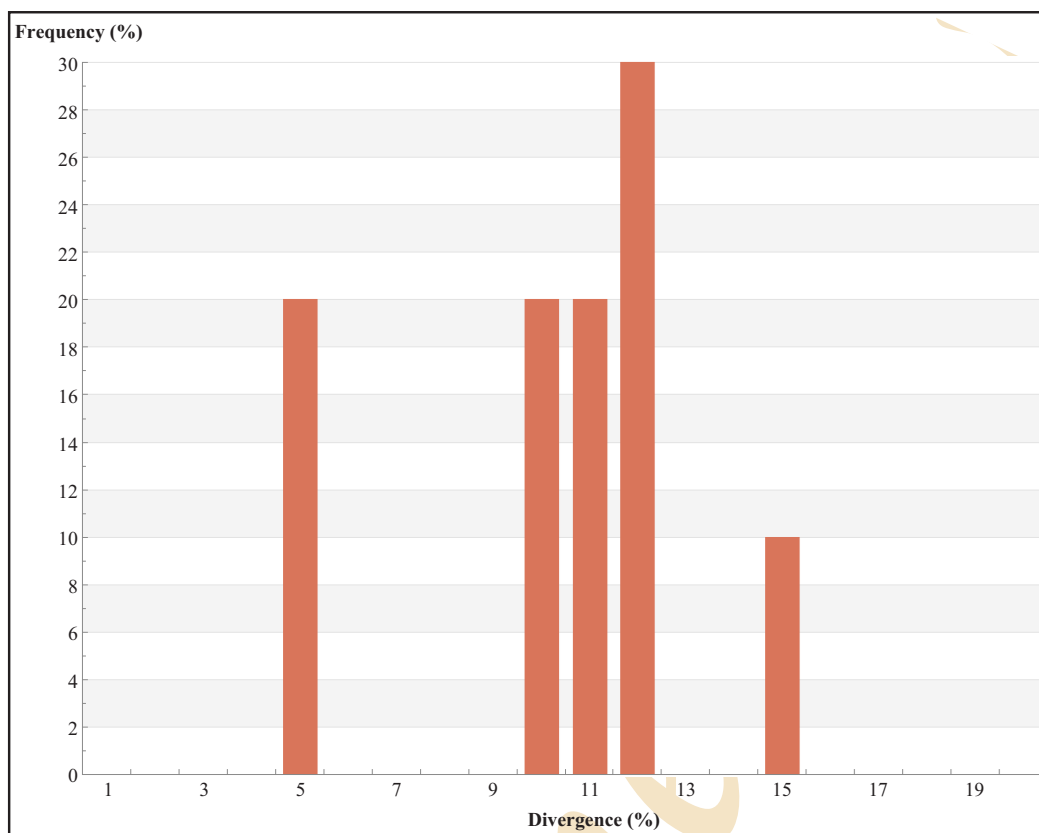
Fig. 4 : The histogram plots the distance to the nearest neighbour

some of samples (haplotypes) to verify the polymorphism in DNA sequence initially detected using the forward primer.

Data analysis : The CHROMAS (version 2.1.1) software (Technelysium Pte Ltd, http://www.technelysium. com.au/chromas.html) was used to display the fluorescence-based DNA sequencing results. A multiple sequence alignments and editing for the forward reactions were done using the CLUSTAL X program (version 2.1, Larkin *et al.*, 2007), and subsequently aligned by eyes. The FaBox program (version 1.41: an online fasta sequence toolbox; users-birc.au.dk/biopv/php/fabox/dnacollapser.php) was used to convert edited multiple sequences into haplotype sequences. The MEGA program version 6.06 (Tamura *et al.*, 2013) was used to perform amino acid translation for all the sequences examined to ensure that no gaps or stop codons were present in the alignment. MEGA was also used to construct a Neighbour-Joining (NJ) tree (Saitou and Nei, 1987) using *Himantura leoparda* (GenBank accession no: JX263320.1) and *Scoliodon laticaudus* (GenBank accession

no: KF899700.1) as outgroup species. Four haplotypes of *Plectropomus maculatus* (GenBank accession no: JN208620.1), *P. leopardus* (GenBank accession no: JQ513291.1), *E. corallicola* (GenBank accession no: JX093908.1) and *E. heniochus* (GenBank accession no: JN208617.1) were included in the analysis to demonstrate the reciprocally monophyletic status between the Epinephelinae. Phylogenetic confidence was estimated by bootstraping (Felsenstein, 1985) with 1000 replicate datasets. The Automatic Barcode Gap Discovery (ABGD) interface web tool (available at http://wwwabi.snv.jussieu.fr/public/ abgd@/abgdweb.html) for species delimitation analysis (Puillandre *et al.*, 2012) was also used in the study. ABGD automatically detects the breaks in distribution of genetic pairwise distances referred to as the "barcode gap" and uses it to partition the data (Puillandre *et al.*, 2012). The BOLD system version 3.1 (Ratnasingham & Hebert, 2007) was used to estimate pairwise genetic distances for intraspecies and intragenus comparisons using the "Distance Summary" command.
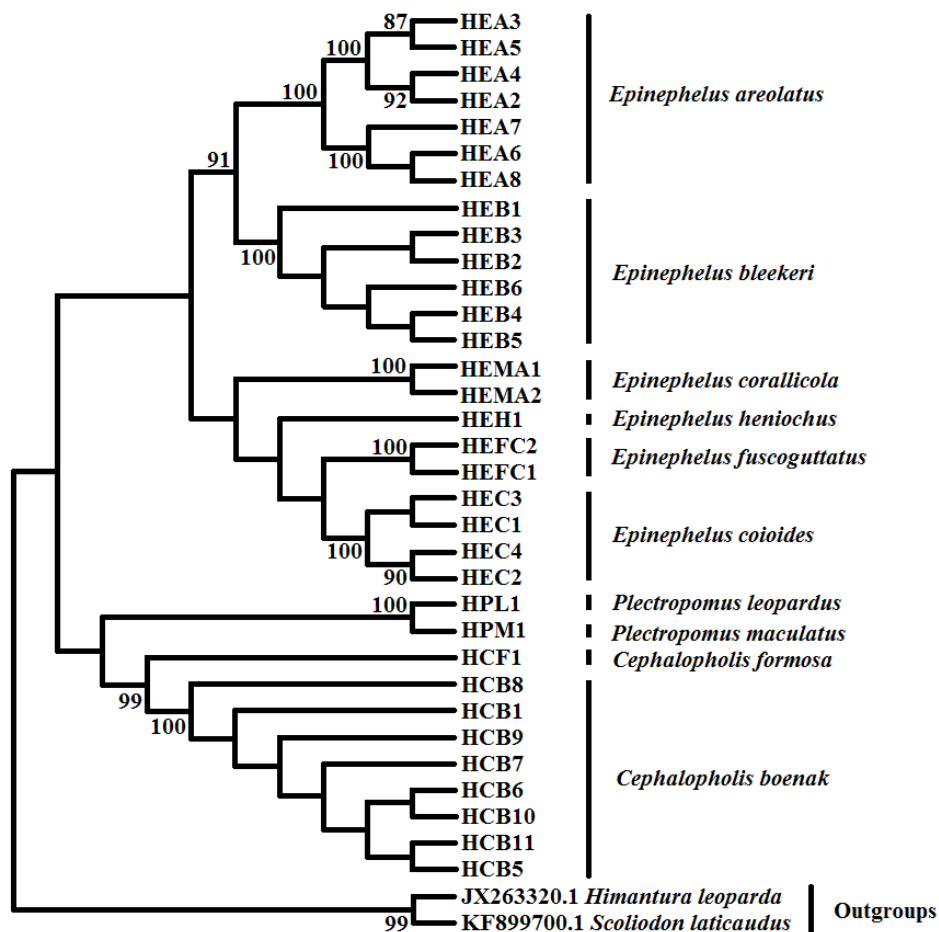
Fig. 5 : Neighbour-Joining (NJ) tree (consensus tree) showing relationships among 63 sequenced grouper (33 haplotype sequences). The number at each node represent the bootstrap value (%) based on 1000 pseudoreplications for NJ analysis

Results and Discussion

Sixty-three partial sequences (629-base pairs) of each of the mitochondrial COI gene were successfully obtained. There were 364 conserved sites, 180 variable sites and 166 parsimony informative sites found from 544-base pairs of aligned sequences. Based on the sequence results, there was no evidence of stop codons, insertion or deletions in any of the amplified sequences indicating that these sequences constituted a functional mitochondrial COI gene fragment. All 63 sequences were subsequently converted into haplotype sequences by using the FaBox interface tool web (server), which reduce the number of data by eliminating the same sequences. Thus, a total of 33 haplotypes were identified and used in the subsequent analysis. All the haplotypes with their respective GenBank accession number are listed in Table 2.

All the haplotypes sequences were subjected to species validation through the BLAST program in order to cross-check their species identity with the available COI sequences from the Genbank and BOLD databases. Results from the BLAST search showed that a total of 55 sequences could be assigned to 10 grouper species (*Cephalopholis formosa, C. boenak, Epinephelus areolatus, E. bleekeri, E. coioides, E. corallicola, E. fuscoguttatus, E. heniochus, Plectropomus leopardus* and *P. maculatus)* from the databases with high percentage identity indices (95% to 100%). However, eight grouper individuals showed discrepancies in their taxonomic identification based on the morphological and COI barcoding results. Accordingly, two individuals morphologically identified as *E. areolatus* were

DNA barcoded as *E. coioides* and *E. bleekeri,* respectively, five individuals were morphologically identified as *E. bleekeri* but DNA barcoded as *E. areolatus,* while one individual was morphologically identified as *E. melanostigma* but DNA barcoded as *P. leopardus.*

This discrepancy observed in the eight grouper samples might be due to the use of 95% ethanol during field samplings, which resulted in several changes in their morphological characteristics (*i.e.* body colours), thus making their identification difficult or confusing. Moreover, Guan *et al*., (2014) stated that many *Epinephelus* species lacked morphological specialisation. The colour and morphological characteristics of many groupers can be quite variable and changes occur as they grow and adapt to different environments (Guan *et al*., 2014).

A similar situation of misidentification has been found in other fish studies such as with Dogfish (Ward *et al.*, 2005), where this species was morphologically identified as two different species, but DNA barcode results identified them as a single species. This discrepancy was due to incorrect identification of the original specimen. Landi *et al*. (2014) stated that there were several identifications based on the morphological characters of Mediterranean marine fish species sequences being checked for similarity with homologous sequences of BOLD-IDS and BLAST databases which resulted in 17 individuals with nine different species. Mismatching species is due to unstable taxonomic status of some species complexes and their synonyms with additions of morphology-based misidentification (Landi *et al*., 2014). Serrao *et al*. (2014) studied two species of Snakehead fish, *Parachanna africana* and *Channa maculate* using barcoding techniques and identified these as two different species, *Parachanna obscura* and *Channa argus*, respectively. This indicates that there are uncertainties in morphological identification or other factors such as hybridization may have naturally occurred (Serrao *et al*., 2014). A similar situation was also observed by Fields *et al.* (2015) in the case of shark misidentification (obtained from GenBank), since a sample of white shark has a 99% sequence identity to blue shark compared to 83% sequence identity to its identified species. Thus, it has been suggested that correct species identification is of particular importance when generating a barcode library, and specimens need to be retained until complete analysis is obtained and also for future studies (Ward *et al*., 2005).

The haplotypes sequences were aligned before being used for computing the matrix of pairwise distances and ran

with P values ranging between 0.001 to 0.1 using Kimura two parameter (Kimura, 1980) (Fig. 2). The histogram of distances showed that there was a clear-cut barcode gap present in the sequences. Meyer and Paulay (2005) stated that DNA barcoding should show a clear separation between intraspecific and interspecific distances which reflects genetic variation within the species and the divergence separating sister species. Hebert *et al*. (2004) suggested that a wide gap between intraspecific and interspecific variation makes a threshold approach promising in DNA barcoding. The within species distribution summarised that the sequence divergence is normalised to reduce any bias present at the species level (Fig. 2). These results were also supported by the ABGD analysis where a clear-cut barcode gap was present in the sequences.

All haplotype sequences were submitted into the BOLD system in order to perform several analyses, which have been provided in the interface tool web. The statistics for nucleotide frequency distribution were different with the highest nucleotide composition of T=30.17% (Standard Error=0.23), followed by C=27.44% (SE=0.23), A=25.24% (SE=0.09), and G=17.15% (SE=0.08). On the other hand, distribution of sequence divergence at each taxonomic level showed a low sequence divergence within-species (1.27% SE=0.02) as compared to within-genus and within-family (14.21% SE=0.01, and 19.17% SE=0.01), respectively. Ward *et al.* (2005) also obtained increasing values of genetic distances which were 0.39% for conspecific, 9.93% for congeneric, and 15.46% for confamilial species in a total of 754 COI sequences, representing 207 species of Australian fish. In another study, Mat Jaafar *et al*., (2012) produced same pattern where genetic divergence progressively increased with higher taxonomic levels. Thus, the results of the present study was in line with the requirements stated in the BOLD system that for barcodes it is necessary to have a low sequence divergence within species as compared to sequence divergence at higher taxonomic levels (Fig. 2).

The barcode gap summarised determination of distance to the nearest neighbour for each species using the MUSCLE alignment program (Edgar, 2004) and Kimura two parameter (Kimura, 1980). The mean intra-specific distances showed a minimum divergence of 0.16% and a maximum divergence of 2.2% with a mean divergence value of 0.81% (Figure 3). Meanwhile, for the nearest neighbour calculation, results showed a minimum distance of 4.47% and maximum distance of 14.08% with a mean value of 9.64% (Fig. 4). The mean intra-specific distance for each species had a low distance compared to nearest neighbour similar to the finding

by Serrao *et al*. (2014). In addition, Table 1 shows the details for species comparisons. N/A was displayed for intra-specific values with species as in singleton. There were four species that had N/A values for their mean and maximum intra-specific values due to low number of sequences present in each species. *E. coioides*, with its nearest neighbour of *E. fuscoguttatus*, exhibited lowest mean intra-specific value of 0.16% (distance to NN=10.26%). Accordingly, both the species live in reef-associated areas and have similar spots and blotches that are brownish in colour (Heemstra and Randall, 1993). Meanwhile, *E. bleekeri,* and *E. areolatus* exhibited highest mean intra-specific value of 2.2% (distance to NN=11.08%). This finding supports their differences in macrohabitats and morphology, where *E. bleekeri* was a demersal oriented fish which lives close to sea floor and has a bluish lower half of caudal fin and no spots, while *E. areolatus* is a reef-associated fish and is grey to whitish in colour with numerous brown spots (Heemstra and Randall, 1993).

All haplotype sequences were further analysed for their interspecific pairwise genetic distances using MEGA. The lowest pairwise distance was found between *P. leopardus* and *P. maculatus* (4.4%), while the highest distance was between *E. bleekeri* and *P. maculatus* (23.5%). This shows close genetic relationship between *P. leopardus* and *P. maculatus,* and thus supported their similar morphological characteristics of having highly similar colour patterns (reddish brown and dark-edge blue spots) all over their body. Juveniles of these species are reportedly found in similar habitats (reef-associated areas) (Heemstra and Randall, 1993).

Phylogenetic analysis using Neighbour-Joining (NJ) method (Fig. 5) showed the presence of two major clades. The first clade consisted of all species from the genus *Epinephelus,* while another clade consisted of species under the genera *Plectropomus* and *Cephalopholis*. Sister clades were found between *C. boenak* with *C. formosa*, *E. areolatus* and *E. bleekeri* and *P. leopardus* with *P. maculatus* with strong bootstrapping confidence levels between 98% to 100%. Ward *et al*. (2005) also found a sister clade relationship between *P. leopardus* and *P. maculatus* in the phylogenetic analysis of Australia fish with lowest mean sequence divergence of 0.19%.

In conclusion, DNA barcoding has proved to be one of the most reliable and accurate methods for confirmation of genetic identity of otherwise morphologically problematic or misidentified species, as observed in the present study. The phylogenetic relationships and genetic divergence at intra and inter-specific levels were also determined in this study. Future research should cover more species and genera of tropical groupers and from other geographical areas in Malaysia and the world.

Acknowledgments

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