Journal of Sustainability Science and Management
Volume 11 *Number* 2, December 2016: 1-10
ISSN: 1823-8556

MOLECULAR PHYLOGENY OF SOME MALAYSIAN GROUPERS (SUBFAMILY: EPINEPHELINAE, FAMILY: SERRANIDAE) INFERRED FROM MITOCHONDRIAL AND NUCLEAR GENE SEQUENCES

NOR SYAKINA NOR RAHIM, YUZINE ESA* AND AZIZ ARSHAD

Department of Aquaculture, Faculty of Agriculture and Institute of Biosciences, Universiti Putra Malaysia, Selangor, Malaysia.

**Corresponding author: yuzine@upm.edu.my*

Abstract: Groupers are known as one of the important marine and estuarine fishes they are widely distributed in the tropical and subtropical open water. Nevertheless, the taxonomy and phylogenetic relationships among wild groupers in Malaysian waters are still poorly studied and need to be further clarified. Thus, this study aimed to elucidate the phylogenetic relationship of wild grouper using samples collected from eight coastal areas in Peninsular Malaysia. Samples were identified manually based on their morphological characteristics. Molecular phylogeny was inferred for subfamily Epinephelinae using a combined analysis of the mitochondrial cytochrome *b* and nuclear Recombination activating gene-1 (RAG-1) genes from 12 species representing three genera of subfamily Epinephelinae. Results from all methods of analyses (Neighbour-joining (NJ), maximum parsimony (MP), maximum likelihood (ML), minimum evolution (ME), and Bayesian analyses (BI)) produced similar tree topologies and strong supported the monophyletic status of the three genera within the subfamily Epinephelinae with genus *Plectropomus* was basal to the other genera. Phylogenetic analyses also divided genus *Epinephelus* into two different clades similar to other previous findings. Nevertheless, several relationships remain unresolved particularly among morphologically similar species (e.g between *E. fario* and *E. longispinis*) and species with limited number of individuals (*E. coioides*). Overall, the findings of this study have managed to provide new insights on the taxonomy, phylogeny and genetic diversity of grouper fishes in Peninsular Malaysia, which are important for their appropriate sustainable management, either for conservation or aquaculture purposes.

Keywords: Epinephelinae, molecular phylogeny, cytochrome *b* gene, mitochondrial DNA.

Introduction

Groupers are marine and estuarine fishes which comprises of approximately 15 genera and 159 species (Heemstra & Randall, 1993; Maggio *et al*., 2005). However, only 59 species from 8 genera were reported from the Malaysia waters (Atan *et al*., 2010). This subfamily is widely distributed over tropical and subtropical open water of Southeast Asia (Tookwinas, 1989). They are carnivorous that feeds on small fish and crustacean (Nagelkerken, 1979) and demonstrate slow growth rate, long life span which enable them to stay in the same place for a long period of time, thus making them vulnerable to over-exploitation (Zhu & Yue, 2008).

Most of the species under subfamily Epinephelinae share similar morphological characteristics (Heemstra & Randall, 1993; Maggio *et al.*, 2005) which make morphological identification and phylogenetic studies difficult or problematic for most of the species (Heemstra & Randall, 1993; Maggio *et al.*, 2005; Ding, *et al*., 2006). Thus, molecular data can assists in data clarifying and resolving taxonomic and phylogeny conflicts among the species (Esa *et al.*, 2012).

Although there were many phylogenetic studies already been conducted on grouper of the subfamily Epinephelinae, the phylogenetic relationships and species identification within this subfamily are still poorly understood or problematic. For example, a phylogenetic study between two closely related genera (*Epinephelus* and *Mycteroperca*) from Eastern Atlantic Seas using mitochondrial

cytochrome *b* (cyt *b*) and 16S ribosomal DNA (Maggio *et al.*, 2005) found very small genetic distances between the two genera. Similar phylogenetic study on 28 species within six genera of subfamily Epinephelinae using cyt *b* gene (Ding *et al*., 2006) found that *Promicrops lanceolatus* and *Cromileptes altivelis* were clustered in *Epinephelus* clade thus they suggested that both species should be amended into genus *Epinephelus*. A molecular phylogeny using multiple genes (16S, 12S, TMO-4C4 and histone H3) found that some of classification of Epinephelinae should be revised (Craig & Hastings, 2006). On the other hand, phylogenetic relationship of groupers from Malaysia done by Baharum & Nurdalila (2011) using cyt *b* gene found very low genetic differences between *Epinephelus fuscoguttatus* and *Epinephelus hexagonatus* which can indicate that they probably belong to the same species.

Nowadays, phylogenetic study has move forward by focusing on combined genes as this approach can increase accuracy and resolution of the results obtained (Fisher-Reid & Wiens, 2011). The cyt *b* gene is the most widely used mitochondrial gene for phylogenetic studies among fishes and are useful for resolving phylogenetic relationships at an intra and inter-specific levels (Kamarudin & Esa, 2009), and this gene is also widely used in combined dataset for phylogenetic study. On the other hand, the RAG-1 gene is a good nuclear gene candidate for inferring phylogenetic relationships in vertebrates including fishes (Wang *et al.*, 2007) by which it contributes in resolving the combined data tree by resolving deep branches (Lavoué & Sullivan, 2004; Fisher-Reid & Weins, 2011). Until currently, studies on the phylogeny using combined genes at the family or subfamily levels of fishes from Malaysia including in the subfamily Epinephelinae is still lacking. Thus, the present study attempted to utilise the combined datasets of both the mitochondrial cyt *b* gene and nuclear recombination activating gene-1 (RAG-1) and to examine the phylogenetic relationship of grouper species within the subfamily Epinephelinae from coastal areas of Peninsular Malaysia.

Materials and Methods

A total of 63 grouper specimens were collected (Table 1) from eight coastal areas namely Selangor, Perak, Melaka, Pahang, Johor, Kedah, Kelantan and Terengganu (Figure 1). The fishes were caught using fishing rod or were purchased directly from local fisherman. Specimens were identified manually using the keys of morphological characteristics provided by Heemstra and Randall (1993) and Atan *et al*. (2010). Specimens were kept frozen in -80°C for long storage while tissue samples (muscle or fin clip) were preserved in 95% ethanol. Total genomic DNA was extracted using Wizard® SV Genomic DNA Purification System kit (Promega Corporation®) and visualised on 1% agarose gels containing GelRed Nucleic Acid gel stain (Biotium), run for approximately 30 minutes at 90 V, and photographed under ultraviolet (UV) trans-illuminator. The isolated DNA was used for further mtDNA analysis.

Figure 1: Map of sampling locations for specimens used in this study. Highlighted is the coastal area that specimens were mostly caught or purchased for this study

Amplification were performed in a final volume of 25 µl containing 50-100 ng of genomic DNA, 5X GoTaq® Flexi buffer, 0.75 mM $MgCl_2$, 0.1 mM of each dNTP, 10 µM for each primer with 0.2 U Taq DNA Polymerase. The Primer pairs that were used in this study were cyb-09H (5' GTGACTTGAAAAACCACCGTTG 3') (Tarbelet *et al.*, 1992) and cyb-07L (5' AATAGGAAGTATCATTCGGGTTTGATG 3') (Tarbelet *et al.*, 1992) for cytochrome *b* gene and Rag1-F1 (5' CTGAGCTGCAGTCAGTACCATAAGATGT 3') and Rag1-R1 (5' CTGAGTCCTTGTGAGCTTCCATRAAYTT 3') (Lopez *et al.,* 2004) for RAG-1 gene.

The thermal cycle began at 94 ˚C for 3 min, followed by 30 cycles of 94 ˚C of denaturation (30 s), 48 ˚C annealing (45 s), and 72 ˚C extension (1 min), with final extension at 72 ˚C for 3 min. Thermal cyclic protocol were optimised to get the optimum condition for each species examined in this study. Subsequently, 5 µl of amplified products were run in electrophoresis with 1% agarose gel and stained with GelRed. Purification was performed using Wizard® SV Gel and PCR Clean-Up System kit (Promega) and were directly sequenced using the BigDye® Terminator v3.0 Cycle Sequencing kit (ACGT) on an ABI 377 automated sequencer (PE Applied Biosystem) using only the forward primer (cyb-09h and Rag-F1).

The DNA sequencing results were displayed using CHROMAS Lite version 2.1 software program. All successful sequences were subjected to Basic Local Alignment Search Tools (Blast) validation of their species identity. Value of Identity (Ident) 98%-100% were taken into consideration to confirm the species. Sequences were subsequently aligned and edited using CLUSTAL X2 version 2.1 (Larkin *et al.*, 2007).

Basic statistic, variables, parsimony informative sites, p-distances and best model were calculated using MEGA 6.06 (Tamura *et al.,* 2013). Molecular phylogenies were constructed using five different methods. Neighbor-joining (NJ) and Minimum Evolution (ME) with correction using Kimura-2 parameter (K2P) model with gamma distribution. Maximum Likelihood (ML) and Maximum Parsimony (MP) were constructed with Hasegawa-Kishino-Yano (HKY) method (Hasegawa *et al*., 1985) with gamma distribution. Maximum likelihood heuristic method was implemented for ML and MP phylogenetic analysis. The phylogenetic analyses were computed based on bootstrap value of 1000 replications which implemented in MEGA 6.06 (Tamura *et al.,* 2013).

The same datasets with addition of partition homogeneity test and AIC parameter were used for Bayesian inference (BI) analysis implemented in MrBayes 3.2.1 (Ronquist *et al.,* 2012). Phylogenetic analyses were performed for both genes separately and concatenated (combined cyt *b* and RAG-1 genes). The best fit model calculated was used for ML and BI analysis. Two partitions (cyt *b* and RAG-1) were considered in BI analysis in a combined data set. However, the disparity index for homogeneity test rejected ($P<0.01$) the null hypothesis that the combination sequences for both two gene dataset have evolved with the same pattern of substitution. The result was expected since the molecular data (mtDNA cyt *b* and nuclear DNA RAG-1) used in this study evolves in different rates (Dolphin *et al*., 2000; Gaines *et al*., 2005; Maggio & Hastings, 2006). Nevertheless, the combined datasets was chosen to be used in the present study.

Results and Discussion

There were 63 grouper individuals collected in this study. Morphological study has identified 12 species belonging to 3 genera (genera *Plectropomus*, *Cephalopholis* and *Epinephelus*). The species were *P. leopardus*, *P. maculatus*, *C. boenak*, *C. formosa, E. bleekeri*, *E. longispinis*, *E. corallicola*, *E. erythrurus*, *E. fuscoguttatus*, *E. coioides*, *E. sexfasciatus* and *E. heniochus* (Table 1).

Table 1: The number of individuals, their locality, IUCN status and accession number for specimens collected used in this study

Species	Locality	N	Haplotype	Accession number	IUCN status
P.leopardus	Terengganu	1	PL1	KR149588	Near threated
	Pahang	1	PL1		
P.maculatus	Pahang	1	PM1	*	Least concern
C.boenak	Terengganu	9	CB1, CB2,CB3, CB5,CB10	KR149576-	Least concern
			CB14,CB18	KR149579	
C.formosa	Terengganu	1	CF1,GH	KR149575	Least concern
	Perak	1			
E.bleekeri	Kelantan	3	EB1,EB2,EB3, EB4,EB6,	KR149580,	Near threated
	Pahang	4	EB19,EB4K, EB5K	KR149581	
	Selangor	1			
	Perak	1			
	Kedah	2			
E.longispinis	Selangor	7	EFA13,EFA15, EFA21,	KR149582-	Least concern
	Perak	1	EFA23,GB	KR149584	
E.corallicola	Johor	2	ECL1,ECL2	KR149585	Data deficient
E.erythrurus	Melaka	3	ERY1,ERY4	*	Data deficient
E.fuscoguttatus	Terengganu	8	EFC20,EFC21, EFC22,EFC23	*	Near threated
	Kedah	2	EFC24,EFC25, EFC28,GW1, GW2		
E.coioides	Perak	1	EC2	*	Near threated
E.sexfasciatus	Kelantan	1	ES1,ES19, ES22,ES23, ES25	KR149586,	Data deficient
	Terengganu	1	ES28,ES29,ES2P	KR149587	
	Selangor	8			
	Perak	2			
E.heniochus	Kelantan	1	EH1,EH4K	*	Data deficient
	Kedah	1			

*Accession number yet to be obtained from Genbank

For the sequence results, a total of 1857 bp of the combined cyt *b* (750 bp) and RAG-1 (1107 bp) genes sequences were aligned where 1404 conserved sites, 453 variable sites and 383 parsimony informative sites. The mean totals nucleotide composition for T, C, G, A among the species were 24.7%, 28.1%, 24.3% and 22.91% respectively. The estimated transition and transversion bias ratio (R) is 4.03. There are small differences between transition/transversion bias values (4.03 to 3.51) to those obtained by Maggio *et al.* (2005). Thus, the combined genes was suitable for studying the phylogenetic relationships among grouper species as it contained both slow and rapidly evolving region (Baharum & Nurdalila, 2012). The pairwise distances showed the highest value were between *P. leopardus* and *E. bleekeri* (14.1%) and *P. leopardus* and *E. corallicola* (14.1%). While, the lowest pairwise distance value was between *E. bleekeri* and *E. longispinis* (1.5%) (Table 2).

A total of 49 haplotypes were identified from the 63 sequences and were subsequently utilized for the phylogenetic tree reconstructions for all methods. All the phylogenetic analyses produced similar tree topology with only slightly differences in their bootstrap values within deeper nodes (Figure 2a and Figure 2b). The results also supported the monophyletic relationships among the subfamily Epinephelinae which were clustered into three monophyletic genera; *Epinephelus*, *Cephalopholis* and *Plectropomus* supported by high bootstrap values, congruent with other previous studies on groupers (Craig *et al.*, 2005; Maggio *et al.*, 2005; Craig & Hastings, 2006). Genus *Plectropomus* was found to be basal to the other two genera which showed that it was earliest genus diverged within the subfamily Epinephelinae, similar with previous results found by Craig *et al*. (2001) and Ding *et al*. (2006).

Table 2: The mean distance between the groups for all eight species analysed under 1000 bootstrap replication. The analyses were conducted using Kimura 2 parameter model (Kimura, 1980) with rate variation of gamma distribution

	Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	*P.leopardus*											
2	*P.maculatus*	0.065										
3	*C.boenak*	0.127	0.107									
4	*C.formosa*	0.129	0.110	0.050								
5	*E.bleekeri*	0.141	0.107	0.094	0.097							
6	*E.longispinis*	0.135	0.105	0.086	0.092	0.015						
7	*E.corallicola*	0.141	0.107	0.092	0.090	0.066	0.060					
8	*E.erythrurus*	0.139	0.106	0.087	0.088	0.065	0.059	0.044				
9	*E.fuscoguttatus*	0.137	0.113	0.110	0.094	0.081	0.071	0.075	0.078			
10	*E.coioides*	0.140	0.113	0.098	0.091	0.076	0.067	0.062	0.059	0.070		
11	*E.sexfasciatus*	0.131	0.099	0.094	0.096	0.080	0.076	0.084	0.072	0.082	0.081	
12	*E.heniochus*	0.138	0.113	0.094	0.096	0.079	0.074	0.081	0.078	0.082	0.084	0.085

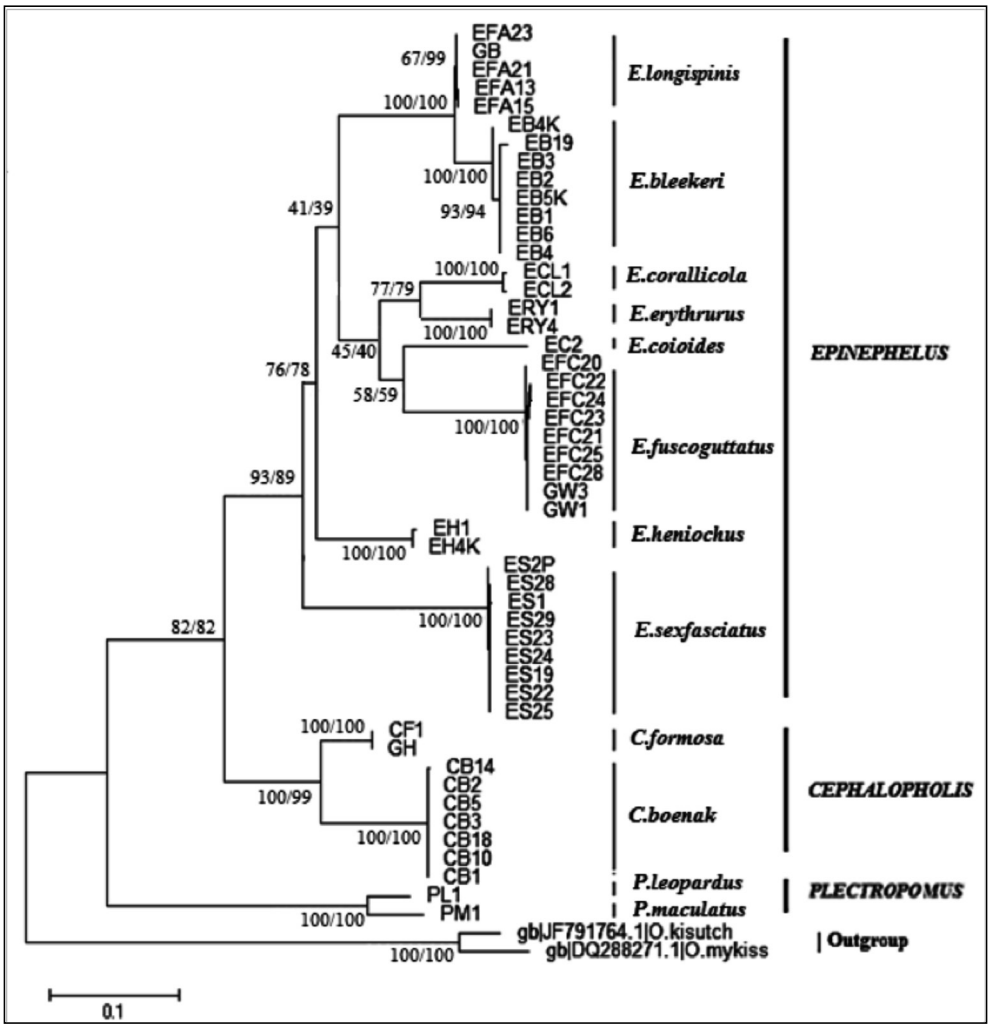

Figure 2a: NJ and ML trees of the ingroup (combined dataset – cyt *b* and RAG-1). Number of bootstrap value are indicated at each nodes below the branch

Phylogenetic analyses of the genus *Epinephelus* divided the sequences into two parallel evolutionary clades supported by high bootstrap values; NJ-82%, ML-82%, ME-82%, MP-95% (some data not shown) and BI-100%. The major clade consisted sequences of *E. longispinis*, *E. bleekeri, E. corallicola*, *E. corallicola, E. erythrurus, E. coioides,* while the smaller clade consisted sequences of only three species; *E. fuscoguttatus,* and *E. heniochus* and *E. sexfasciatus*, with basal taxa for that group was *E. sexfasciatus*. This findings were similar with those reported by Craig *et al.* (2001) and Ding *et al.* (2006). Ding *et al.* (2006) discussed that divergence from the ancestor of *Epinephelus* forming two clades might happen during early stage of evolution. However, further molecular evolutionary studies are required to resolve the issue. Besides that, the current result showed that the *E. bleekeri* and *E. longispinis* have little pairwise genetic distance (1.5%) between them compared to the other species. This supported the fact that they are morphologically similar and almost exhibited lack of distinct species-specific characteristics (Ding *et al.*, 2006). In

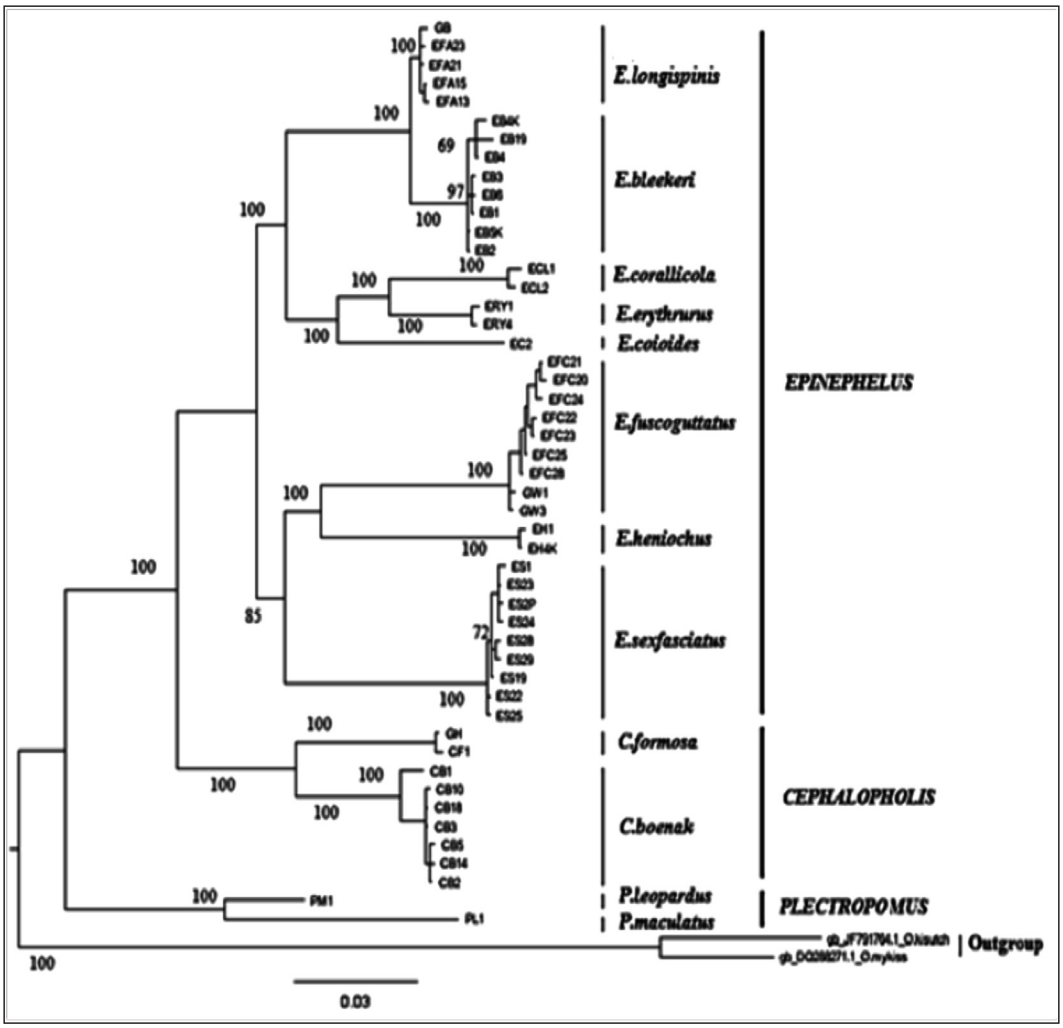

Figure 2b: BI tree on the concatenated dataset (cyt *b* and RAG-1 genes) used in the present study. Bootstrap values were indicated for each nodes

addition, minor differences in the deeper node positioning of *E. coioides* was found based on BI analyses. Accordingly, the other four methods grouped *E. coioides* together with *E. fuscoguttatus E. fuscoguttatus* (NJ-58, ML-59, ME-57, and MP-60) but BI tree clustered *E. coioides* together with *E. corallicola* and *E. erythrurus* in the same clade with high posterior probability support (BI-100).

Another interesting finding of this study was the taxonomy confusion between morphologically similar species and the lacked of molecular data for species of the genus Epinephelus, especially from this region in the Genbank database. *E. fario* was largely regarded as a false name used for *E. longispinis* and a few authors suggested that they should be taxonomically identified as the same species due to their morphological similarity (Heemstra & Randall, 1993). The BLAST results of the current study showed that the *E. longispinis* (KR149582) cyt *b* sequences were highly identical (99-100% identity index) to *E. fario* (DQ486931.1) sequence from the Genbank database, thus supporting their high genetic similarity. Nevetheless, previous studies done

by Guan *et al.* (2014) and Sachithanandam *et al.* (2012) using mitochondrial CO1 sequences suggested that *E. fario* and *E. longispinis* were two genetically different species and they were grouped into two separate clades in their phylogenetic tree analysis. Thus, the taxonomy and phylogeny of the two species could not be resolved from the current study and more effort are needed to shed light on the issue (Fennessy *et al*., 2008; Bailly, 2015).

Conclusion

The present phylogenetic study using a combined mitochondrial cyt *b* and nuclear RAG-1 genes has managed to provide useful insights into phylogenetic relationships and species identification of wild groupers of the subfamily *Epinephelinae* from Peninsular Malaysia. The present results are generally congruent with previous studies on the phylogeny of groupers with minor differences in positioning and pairwise genetic distances values for some species. The reciprocally monophyletic status within the three genera in the subfamily *Epinephelinae* was strongly supported but relationships between a few species was still not fully resolved. We recommended that future studies should be continued in order to resolve the remaining phylogeny and taxonomy issues within the groupers especially, for groups which lacked morphologically characteristics (e.g. between *E. longispinis* and *E. fario* in this present study). This can be done by using larger sample size and more sampling locations throughout Malaysia, inclusion of more grouper species, and utilisation of longer DNA sequences or complete mitochondrial genome sequence analyses. Overall, the findings of this study should contribute to better understanding on the taxonomy, phylogenetic and genetic diversity which would lead to appropriate sustainable management of grouper fishes in Peninsular Malaysia, either for conservation or selection of broodstock from species of interest for aquaculture purposes.

Acknowledgements

The authors would like to thank all the colleagues of Fish Genetics Laboratory, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia and Institute of Biosciences (IBS) and everyone involved in filed samplings for their support and assistance in completion of this study.

References

Atan, Y., Jaafar, H., & Majid, A. R. A. (2010). *Ikan Laut Malaysia: Glosari Nama Sahih Species Ikan.* Kuala Lumpur: Dewan Bahasa dan Pustaka.

Baharum, S. N., & Nurdalila, A. A. (2011). Phylogenetic Relationships of *Epinephelus fuscoguttatus* and *Epinephelus hexagonatus* Inferred from Mitochondrial Cytochrome b gene Sequences Using Bioinformatics Tools. *International Journal of Bioscience, Biochemicals & Bioinformatics,* 1(1): 47-52.

Baharum, S. N., & Nurdalila, A. A. (2012). Application of 16s rDNA and Cytochrome b Ribosomal Markers in Studies of the Lineage and Fish Populations Structure of Aquatic Species. *Molecular Biology Reports,* 3: 5225-5232.

Bailly, N. (2015). *Epinephelus fario* (Thuberg, 1793). In: Froese, R. and D. Pauly. Editors (2015) FishBase. Accessed through: World Register of Marine Species. Retrieved from http://www.marinespecies.org/aphia.php?p=taxdetails&id=218208 on 19 April 2015.

Craig, M. T., Pondella, D. J., Frank, J. P. C., & Hafner, J. C. (2001). On the Status of Serranid Fish Genus *Epinephelus*: Evidence for Paraphyly Based Upon 16S rDNA Sequences. *Molecular Phylogenetics & Evolution,* 14: 121-398.

Craig, M. T., & Hastings, P. A. (2006). A Molecular Phylogeny of the Groupers of the Subfamily Epinephelinae (Serranidae) with a Revised Classification of the Epinephelini.

Ichthyological Research, 54: 1-17.

Ding, S., Zhuang, X., Guo, F., Wang, J., Su, Y., Zhang, Q., & Li, Q. (2006). Molecular Phylogenetic Relationship of China Seas Groupers Based on Cytochrome b gene Fragment Sequences. *Science China Life Sciences,* 49(3): 235-242.

Dolphin, K., Belshaw, R., Orme, C. D. L., & Quicke, D. L. J. (2000). Noise and Incongruence: Interpreting Results of the Incongruence Length Difference Test. *Molecular Phylogenetics & Evolution,* 17: 401-406.

Esa, Y. B., Siraj, S. S., Daud, S. K., Rahim, K. A. A., Japning, J. R. R., & Soon, G. T. (2008). Mitochondrial DNA Diversity of *Tor tambroides* Valenciennes (Cyprinidae) from Five Natural Populations in Malaysia. *Zoological Studies,* 47(3): 360-367.

Fennessy, S., Myers, R., & Pollard, D. (2008). *Epinephelus longispinis*. The IUCN Red List of Threatened Species. Version 2014.3. Retrieved from IUCN Red List at www.iucnredlist.org.

Fisher-Reid, M. C., & Weins, J. J. (2011). What are the Consequences of Combining Nuclear and Mitochondrial Data for Phylogenetic Analysis? Lessons from Plethodon salamanders and 13 other vertebrate clades. *BMC Evolutionary Biol*ogy, 11: 300-320.

Gaines, C. A., Hare, M. P., Beck, S. E., & Rosenbaum, H. C. (2005). *Nuclear Markers Confirm Taxonomic Status and Relationships among Highly Endangered and Closely Related Right Whale Species. Proceedings of the Royal Society of London, Series B,* 272: 533-542.

Guan, S., Liu, H., Zheng, Y., Yu, D., Nie, A., & Liu, M. (2014). Inference of Phylogenetics and Evolution of *Epinephelus septemfasciatus* and 48 Other Species of *Epinephelus* Genus Using Mitochondrial CO1 Fragment Sequences. *Pakistan Journal of Zoology,* 46(1): 67-74.

Hasegawa, M., Kishino, H., & Yano, T. (1985). Dating the Human-ape Split by a Molecular Clock of Mitochondrial dna. *Journal of Molecular Evolution,* 22: 160-174.

Heemstra, P. C., & Randall, J. E. (1993). *Groupers of the World, FAO Species Cataloge*. Rome. FAO.

Kamarudin, R., & Esa, Y. (2009). Phylogeny and Phylogeography of *Barbonymus schwanenfeldii* (Cyprinidae) from Malaysia Inferred Using Partial Cytochrome b mtDNA Gene. *Journal of Tropical Biology & Conservation,* 5: 1-13.

Kimura, M. (1980). A Simple Method for Estimating Evolutionary Rate of Base Substitutions through Comparative Studies of Nucleotide Sequences. *Journal of Molecular Evolution,* 16: 11-120.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., William, A., Lopez, R., Thompson, J. D., Gibson, T. J., & Higgins, D. G. (2007). Clustal W and Clustal X Version 2.0. *Bioinformatics,* 23: 2947-2948.

Lavoué, S., & Sullivan, J. P. (2004). Simultaneous Analysis of Five Molecular Markers Provides a Well-supported Phylogenetic Hypothesis for the Living Bony-tongue Fishes (Osteoglossomopha: Teleostei). *Molecular Phylogenetics & Evolution,* 33: 171-185.

López, J. A., Chen, W. J., & Ortí, G. (2004). Esociform Phylogeny. *Copeia* 3: 449-564.

Maggio, T., Andaloro, F., Hemida, F., & Arculeo, M. (2005). A Molecular Analysis of Some Eastern Atlantic Grouper from the *Epinephelus* and *Myctoperca* Genus. *Journal of Experimental Marine Biology & Ecology,* 32: 83-92.

Maggio, T., & Hastings, P. A. (2006). A Molecular Phylogeny of the Groupers of the Subfamily Epinephelinae (Serranidae) with a Revised Classification of the Epinephelini. *Ichthyological Research*, 54: 1-17.

Nagelkerken, W. P. (1979). Biology of the Grysby, *Epinephelus cruentatus* of the Coral Reef of Curacao. *Studies Faun Curacao,* 60: 1-118.

Ronquist, F., Teslenko, M., Van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. L., & Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice across a Large Model Space. *Systematic Biology*, 61: 539-542.

Sachithanandam, V., Mohan, P. M., Muruganandam, N., Chaaithanya, I. K., Dhivya, P., & Baskara, R. (2012). DNA Barcoding, Phylogenetic Study of epinephelus spp. from Andaman Coastal Region, India. *Indian Journal of Geomarine Sciences,* 41(3): 203-211.

Tarbelet, P. A., Meyer, A., & Bouvet, J. (1992). Unsually Large Mitochondrial Variation in Populations of Blue Tit, *Parus caereleus*. *Molecular Ecology,* 1: 27-36.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Moecular Biology & Evolution,* 30: 2725-2729.

Tokwinas, S. (1989). Review of Knowledge on Grouper Aquaculture in Southeast Asia. In: *Proceedings of Advances in Tropical Aquaculture*. 20 February–4 March 1989, Tahiti: AQUACOP IFREMER, Actes de Colloque, 429-435.

Wang, X., Li, J., & He, S. (2007). Molecular Evidence for the Monophyly of East Asian Groups of Cyprinidae (Teleostei: Cypriniformes) Derived from the Nuclear Recombination Activating Gene 2 Sequences. *Molecular Phylogenetics & Evolution*, 42(1): 157-170.

Zhu, Z. Y., & Yue, G. H. (2008). The Complete Mitochondrial Genome of Red Grouper (*Plectropomus leopardus*) and Its Applications in Identification of Grouper Species. *Aquaculture*, 276(1-4): 44-49.