The Journal of **Macro**Trends in Applied Science

MACROJOURNALS

Genetic Identification of Shark From Malay Peninsular Using Cytochrome Oxidase Subunit 1 Gene

Badrul Munir Md-Zain, Nur Illi Ayuni Abdul Jalil, Noor Haslina Masstor, Nor Aifat Rahman, Salmah Yaakop and Abdullah Samat

School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600, Selangor, Malaysia

Abstract

E A molecular study was carried out to identify shark species in Malay Peninsular. using Cytochrome Oxidase Subunit 1 (COI). The main objective of this study was to determine the shark species identity from local markets and freshly caught by fishermen. Total DNA were amplified using Polymerase Chain Reaction for their COI sequences. Samples were sequenced and the phylogenetic tree was reconstructed. Two species were selected as the outgroup which were Chimaera monstrosa and Urobatis halleri. A total of 750 base pair was fully obtained with 11 shark species were successively identified namely Chiloscyllium griseum, Chilosyllium indicum, Chiloscyllium punctatum, Carcharhinus sorrah, Carcharhinus sealei, Rhizoprionodon acutus, Galeocerdo cuvier, Sphyrna Lewini, Hemigaleus microstoma, Centrophorus moluccensis and Squalus montalbani. Phylogenetic tree portrayed the shark species relationships and identity according to their order Carcharhiniformes, Orectolobiformes and Squaliformes. This study showed the COI ability for Malaysian shark genetic identification at species level.

Keywords: *Shark, Species identification, DNA barcode, Phylogenetic relationships*

1. Introduction:

A total number of 110 species of Elasmobranchs has been recorded in Malaysia and Brunei (Yano et al. 2005). Sharks dominated the numbers with 56 followed by rays (52) and chimaeras (2). The most recent study added seven new species (Last et al. 2010). Despite records from several species inventory studies in Southeast Asia, the actual biodiversity Elasmobranchs status

still remain lack of information. A global concern is the impact of fishing on chondrichthyan stocks around the world. It is seldom for whole sharks to be sold commercially (Clarke et al. 2006; Liu et al. 2013). Instead, they are finned at sea whereby the fins are kept while the bodies of the sharks are discarded. Later, the fins go through a drying process prior to being sold to wholesalers (Sembiring et al. 2014).

Since the past few decades, studies on animal taxonomy and systematic have often employed mitochondrial DNA (mtDNA) as genetic marker (Liedigk et al. 2015). One part of the mtDNA is the cytochrome oxidase subunit I gene (COI) which possesses a few extra characteristics that make it a highly favoured molecular marker for systematic studies. Furthermore, COI is highly conserved and variable regions that make this gene the most suitable marker for genetic identification at species level and remain as popular for DNA barcoding. Species identification through DNA barcoding is achieved by retrieving a short sequence of COI DNA sequences that is referred to as the barcode which is from a standard part of the genome from the investigated specimen (Hajibabaei et al. 2007). The barcode from unknown species is then be compared with a library of reference barcode sequences where the identity of the individuals' sequences are known. In addition, the role of COI gene in identifying genetic samples at species level is important for taxanomy, systematic and biodiversity studies (Hajibabaei et al. 2007). Few previous molecular studies are available to portray shark diversity in Malaysia (Masstor et al. 2016). Thus, this study was carried out in order to determine shark species diversity in Malay Peninsular caught by local fishermen using COI gene as genetic marker for species identification.

2. Materials and methods:

2.1 Sample Collection and DNA Tissue Extraction

Shark tissue samples were obtained from freshly caught animals and preserved in 95% ethanol. All of the tissue samples were collected from local markets and also from fishermen in states of Penang, Terengganu, Pahang, Johor, and Kedah. Additional DNA sequences were obtained from GenBank for comparison. Mitochondrial DNA was isolated from 25 mg of muscle tissue using Invisorb® Spin Tissue Mini Kit, following the manufacturer's suggested protocol.

2.2 DNA Amplification

A pair set of primers following Ward et al. (2005) were used in the PCR for COI amplification (FishF2 5'TCGACTAATCATAAAGATATCGGCAC3'and
FishR2 5'ACTTCAGGGTGACCGAAGAATCAGAA3'). Thermal cycling for the COI locus consisted of an initial step at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 seconds (sec), primer annealing at 47-50°C for 30 sec, extension at 60°C for 60 sec, with a final extension at 72°C for 7 min. The DNA extract (3 µl) was amplified in a final volume of 25 ul, consisting of 12.5 PCR Mix, 20 pmol/ul each primer (1.0 µl) and 7.5 µl ddH_2O. Amplified products were analyzed by electrophoresis in 1.5% agarose gel. PCR products were purified and all the successfully amplified PCR products were sent to an accredited laboratory, 1st Base Laboratories Sdn Bhd for sequencing services.

2.3 Data analysis

DNA sequence editing was performed with BioEdit version 7.0.2 and visually checked. The sequences were then edited on notepad and aligned using ClustalW that incorporated into the MEGA6 software. Each individual sequence was compared with other fish mitochondrial genomes using NCBI BLAST (Altschul et al. 1997) to confirm its identity of the DNA fragments. For construction of Neighbour-Joining (NJ) tree, the distance model Kimura two-parameter (K2P) was employed (Kimura 1980). This phylogenetic analysis was conducted using Phylogeny Analysis Using Parsimony (PAUP) version 4.0 (Swofford 2002). Bootstrap analysis was performed using 1000 replications to assess robustness of branching patterns and analysis reliability.

3. Results and Discussion:

From 20 genetic samples, 14 of them produced good DNA sequences for phylogenetic analysis. Figure 1 indicated PCR products with annealing temperature at 55ºC that visualized in 1.5% agarose gel. Using a 100 bp ladder as a molecular marker, the samples were shown to be amplified at the size of 750 bp. The PCR products of the samples were stored in the freezer at a temperature of -20 ºC in order to prevent DNA degradation from occurring. All PCR products were sent for purification and sequencing at an external laboratory. The selection of which sequences to be used in analysis was determined by viewing the chromatograms of the sequences. Sequences that have clear nucleotide peaks and low background noise (Figure 2) were chosen for analysis.

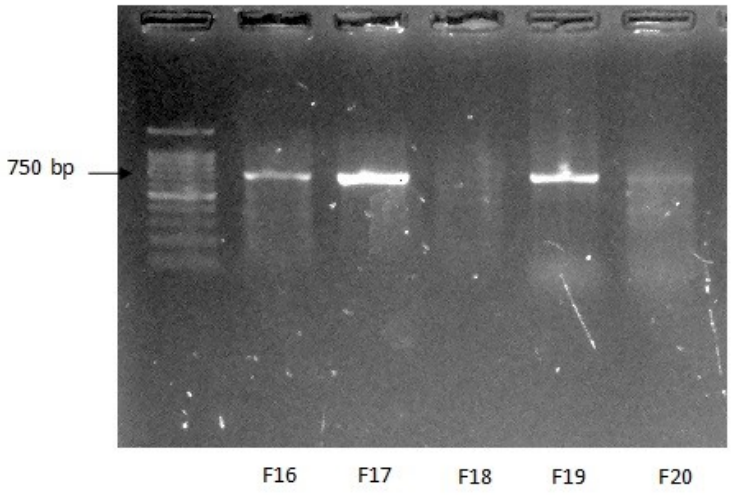


Figure 1. Optimisation of annealing temperature at 55ºC.

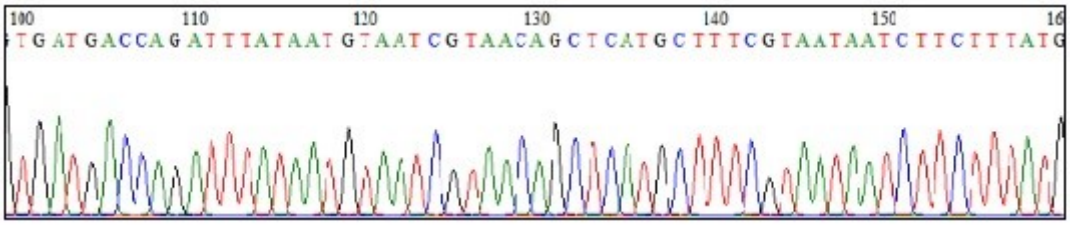
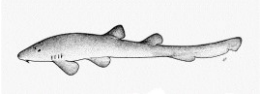
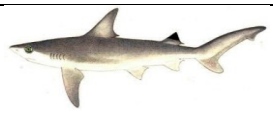


Figure 2. DNA chromatogram for COI with good base peaks

The DNA sequences were confirmed for their respective species using NCBI BLAST online database. *Chimaera monstrosa and Urobatis halleri* DNA sequences were added as the outgroup. After confirming their species, their conservation status identity was retrieved from IUCN red data list. Phylogenetic analyses revealed 11 sharks species with confirmed their genetic identification (*Chiloscyllium griseum*, *Carcharhinus sorrah*, *Chiloscyllium punctatum*, *Rhizoprionodon acutus*, *Chiloscyllium indicum*, *Galeocerdo cuvier*, *Centrophorus moluccensis*, *Sphyrna lewini*, *Squalus montalbani*, *Hemigaleus microstoma*, and *Carcharhinus sealei* (Table 1).

Table1. List of identified shark species, sample code, local name and IUCN conservation status

Photo	Species, code, local name, IUCN status	Photo	Species, code, local name, IUCN status
	*Chiloscyllium griseum* F01, F09, F22 Yu Cicak Gempal Near Threatened		*Sphyrna lewini* F17 Yu Tukul Sanggul Endangered
	*Carcharhinus sorrah* F07, F21 Yu Kepak Hitam Near Threatened		*Squalus montalbani* F18 Yu Taji Vulnerable
	*Rhizoprionodon acutus* F08 Yu Muncung Susu Least Concern		*Hemigaleus microstoma* F19 Yu Bintik Putih Vulnerable
	*Chiloscyllium indicum* F11 Yu Cicak Tembaga Near Threatened		*Chiloscyllium punctatum* F01 Yu Cicak Insang Putih Near Threatened
	*Galeocerdo cuvier* F12 Yu Tenggiri Near Threatened		*Carcharhinus sealei* F23 Yu Pasir Near Threatened
	*Centrophorus moluccensis* F16 Yu Duri Data Deficient		

Based on IUCN red list of threatened species, it was found that *Sphyrna lewini* was the only shark listed as endangered. Six species was listed as near threatened (*Chiloscyllium griseum*, *Chiloscyllium indicum, Chiloscyllium punctatum, Carcharhinus sorrah, Galeocerdo cuvier, Carcharhinus sealei*), 2 vulnerable (*Squalus montalbani, Hemigaleus microstoma)*, 1 data deficient (*Centrophorus moluccensis)* and 1 least concern (*Rhizoprionodon acutus*). *Sphyrna*

lewini, hammerhead sharks, members of the family Sphyrnidae, is also being sold at local market need urgent conservation action as it is listed as endangered shark species in Malay Peninsular. Currently in Malay Peninsular, there are no active conservation actions taken to control the shark trading.

Neighbor Joining (NJ) tree (Figure 3) portrayed the phylogenetic relationships among 11 sharks species with 2 outgroup for comparison. *Chiloscyllium indicum*, *Chiloscyllium punctatum* and *Chiloscyllium griseum* were group in a single monophyletic group under squaliformes with 100% bootstrap values (Masstor et al. 2014). In addition, Carcharhinidae, Sphyrnidae and Hemigalidae have closed genetic relationships eventhough the Famili Sphyrnidae has paraphyletic towards Carcharhinidae (Velez-Zuazo & Agnarsson 2010).

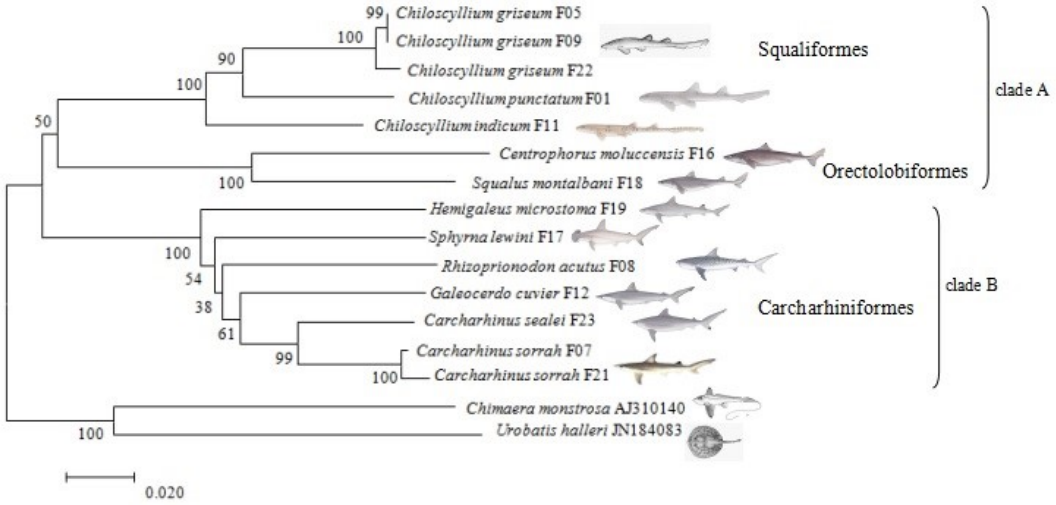


Figure 3. NJ tree depicted phylogenetic relationships among taxon sampled

4. Conclusion:

Our study indicated that the COI gene is reliable tools to investigate the genetic identification of 11 shark species in Malay Peninsular and their Famili phylogenetic relationships. This study could be improved by increasing more taxon sampling and localities.

Acknowledgments
The authors acknowledge Universiti Kebangsaan Malaysia and the Southeast Asian Fisheries Development Center (SEAFDEC), Malaysia, for providing necessary funding, facilities, and assistance. This research was supported by Grants FRGS/1/2015/SG03/UKM/02/1, GUP-2017-087, DLP-2013-006, AP-2015-004, and UKM-GUP-PLW-08-11-046.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids research* 25(17): 3389-3402.

Clarke, S.C., McAllister, M.K., Milner-Gulland, E.J., Kirkwood, G.P., Michielsens, C.G., Agnew, D.J., Pikitch, E.K., Nakano, H. and Shivji, M.S., 2006. Global estimates of shark catches using trade records from commercial markets. *Ecology letters* 9(10): 1115-1126.

Hajibabaei, M., Singer, G.A., Hebert, P.D. and Hickey, D.A., 2007. DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *TRENDS in Genetics* 23(4): 167-172.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16(2): 111-120.

Last PR, White WT, Pogonoski JJ. 2010. Descriptions of new sharks and rays from Borneo. CSIRO Marine & Atmospheric Research Paper No 032.

Liedigk, R., Kolleck, J., Böker, K.O., Meijaard, E., Md-Zain, B.M., Abdul-Latiff, M.A.B., Ampeng, A., Lakim, M., Abdul-Patah, P., Tosi, A.J., Brameier, M., Zinner, D. & Roos, C. 2015. Mitogenomic phylogeny of the common longtailed macaque (*Macaca fascicularis fascicularis*). *BMC Genomics* 16: 222.

Liu, S.Y.V., Chan, C.L.C., Lin, O., Hu, C.S. and Chen, C.A., 2013. DNA barcoding of shark meats identify species composition and CITES-listed species from the markets in Taiwan. *PLoS One* 8(11): p.e79373.

Masstor, N.H., Samat, A., Shukor, M.N. and Md-Zain, B.M. (2014). Molecular Phylogeny of the Bamboo Sharks (*Chiloscyllium* spp.). *BioMed Research International* Volume 2014: Article ID 213896.

Sembiring, A., Pertiwi, N.P.D., Mahardini, A., Wulandari, R., Kurniasih, E.M., Kuncoro, A.W., Cahyani, N.D., Anggoro, A.W., Ulfa, M., Madduppa, H. and Carpenter, K.E., 2015. DNA barcoding reveals targeted fisheries for endangered sharks in Indonesia. *Fisheries Research* 164: 130-134.

Swofford, D.L. 2002. Phylogenetic analysis using Parsimony. Massachusetts: Sinauer Associates.

Vélez-Zuazo, X. and Agnarsson, I., 2011. Shark tales: a molecular species-level phylogeny of sharks (Selachimorpha, Chondrichthyes). *Molecular Phylogenetics and Evolution* 58(2): 207-217.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R. and Hebert, P.D., 2005. DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* 360(1462): 1847-1857.

Yano, K., Ahmad, A., Gambang, A.C., Idris, A.H., Solahuddin, A.R. and Aznan, Z., 2005. *Sharks and rays of Malaysia and Brunei Darussalam.* Marine Fishery Resources Development and Managment Department, Southeast Asian Fisheries Development Center.