

SPECIES CHECKLIST AND DNA BARCODING OF CEPHALOPODS FROM PULAU TINGGI, JOHOR, MALAYSIA

NUR AMIRA SHAFIQA ROSLI¹, MUHAMMAD ASYRAF AZAHAR¹, NOR SHAHIDA AB RAHMAN¹, NUR SABRINA BADRUL HISHAM^{1,2}, SITI NAJIHAH SOLEHIN^{1,2}, 'AISYAH MOHAMED REHAN³, MD-ZAIRI ZAINAL⁴, MUHAMMAD ABU BAKAR ABDUL LATIFF^{1,5,6} AND KAMARUL RAHIM KAMARUDIN^{1,2*}

¹Department of Technology and Natural Resources, Faculty of Applied Sciences and Technology, Universiti Tun Hussein Onn Malaysia (Pagoh Campus), Pagoh Higher Education Hub, KM 1, Jalan Panchor, 84600 Muar, Johor, Malaysia.

²Centre of Research for Sustainable Uses of Natural Resources, Faculty of Applied Sciences and Technology, Universiti Tun Hussein Onn Malaysia (Pagoh Campus), Pagoh Higher Education Hub, KM 1, Jalan Panchor, 84600 Muar, Johor, Malaysia. ³Department of Chemical Engineering Technology, Faculty of Engineering Technology, Universiti Tun Hussein Onn Malaysia (Pagoh Campus), Pagoh Higher Education Hub, KM 1, Jalan Panchor, 84600 Muar, Johor, Malaysia. ⁴Shaz Resort Pulau Tinggi Sdn. Bhd., Lot 44, Kampung Penaga, Pulau Tinggi, 86800 Mersing, Johor, Malaysia. ⁵Environmental Management and Conservation Research Unit (eNCORe), Faculty of Applied Sciences and Technology, Universiti Tun Hussein Onn Malaysia (Pagoh Campus), 84600 Muar, Johor, Malaysia. ⁶Institute of Tropical Biodiversity and Sustainable Development, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia.

*Corresponding author: kamarulr@uthm.edu.my

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Abstract: Cephalopods are one of the protein sources in fisheries and their unique look inspires humans in many aspects from arts to engineering. However, there was a lack of studies on cephalopod species presence and genetics in Malaysia especially in the waters of Johor. Therefore, this study aimed to document the species checklist of coastal cephalopods in Pulau Tinggi, Mersing, Johor. The sampling was limited to the coastal area of Kampung Tanjung Balang, Pulau Tinggi. The specimens of cephalopods were obtained through handpicking (for the octopus) and from the local people (for the squids). In this study, the initial species identification of the cephalopod specimens was done based on their morphological characteristics. Their tissue samples were then prepared for DNA sequencing for further species identification using cytochrome c oxidase I (COI) gene sequences. The morphological identification and the Basic Local Alignment Search Tool (BLAST) analysis results suggested their species status as *Octopus laqueus*, *Sepioteuthis lessoniana* and *Uroteuthis (Photololigo) chinensis*. In conclusion, this study has contributed to the latest update on the checklist of cephalopod species in Pulau Tinggi. Furthermore, adding the COI gene sequences of the local cephalopods in the GenBank database (Accession No.: MN711655-MN711658) will act as a new resource for future research on cephalopod species.

Keywords: Cephalopods, Pulau Tinggi, species checklist, DNA barcoding, COI gene.

Introduction

Malaysia is a tropical country located in the Indo-Pacific region, where biodiversity is probably the richest and most culturally diverse in the world (Biodiversity in Indo-Pacific, 2019). Being one of the megadiverse countries, Malaysia is considered to have the greatest diversity of marine species in the world including the cephalopods and its continental shelf is one of the largest within the tropical

region (Briggs, 1974). The Seribuat archipelago comprises a complex of islands in the southeast of Peninsular Malaysia, where up to 62 islands of different sizes have a distinct richness of biodiversity [Malaysian Nature Society (MNS), 2019]. Most of the islands are a part of Johor, thus, the richness of marine species recorded in Johor water is about 200 species, comprising corals, fishes, molluscs and marine mammals (JMarine, 2019).

Cephalopods, the head-footed animals are unusual molluscs with reduced or absence of shell and complex nervous system that leads to them being the subject of physiology (Hanlon & Messenger, 2018) and neuroscience research (Gilbert *et al.*, 1990). Appeared as a different class over 450 million years ago, they were described through fossil records by their ancestors comprising of heavily shelled nautilus and ammonites but the modern forms that we have known today (nautilus, octopus, cuttlefish and squid) arose only less than 100 million years ago (Boyle & Rodhouse, 2005). Being exclusively marine, they can be found worldwide including in Johor, Malaysia, in every marine habitat of temperate and tropical seas from intertidal to abyssal depths with benthic to pelagic life forms (Pissarra, 2017).

Their wide distribution across the globe depicts the diversity of cephalopod species in adapting to all marine ecosystems, the ability to survive their predators and their flexibility in acquiring prey (Villanueva *et al.*, 2017). Around 845 species have been described worldwide (Hoving *et al.*, 2014); among them, 486 are coastal cephalopods with the highest species richness in the Pacific Ocean, as many as 212 species (Pissarra, 2017). In the South China Sea, 153 species of cephalopods cover Taiwan Strait in the north, the equator to the south and Borneo and the western Philippines to the east (Norman *et al.*, 2016).

DNA barcoding has become an effective tool, particularly in species identification ever since Professor Paul Hebert presented the concept and his colleagues in 2003, where the mitochondrial genome was favoured over the nuclear genome for the analysis and specifically the cytochrome c oxidase I gene (COI) were chosen as the barcode for animals (Hebert *et al.*, 2003). The universal DNA primers used for the identification of cephalopods are LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') as this pair has shown to amplify the fragment of COI across a vast array of invertebrates (Folmer *et al.*, 1994)

consistently. Furthermore, the identification of cephalopods can be made easier by referring to the genetic sequence database of the Barcode of Life Project and GenBank. The Cephalopod International Advisory Council (CIAC) has suggested the concept of a Barcode of Life Database (BoLD) for cephalopods and it can be a credible reference for identification when traditional ways are unable to identify or unavailable (Strugnell & Lindgren, 2007). The presence of this characteristic is essential for the identification of cephalopods, especially if they are in poor shape or from the contents of the stomach, eggs, immature forms, excreta or if they have been seriously damaged (Strugnell & Lindgren, 2007). The GenBank is also helpful in identifying the unknown gene sequence of cephalopods by comparing it with the gene sequence in the database (Weir, 2014).

It is crucial to first identify the cephalopod species through morphological identification or DNA barcode, so, we can focus on the life cycle, behaviour, breeding and feeding grounds, and species distribution. Once its behaviour is understood, the management plan can be devised, targeting its hotspots and assessment for the benefit of fisheries stock as well as the status of its population. Therefore, the focal point of this study is to identify species of cephalopods present in Pulau Tinggi, Mersing and Johor using morphological identification and DNA barcoding method. The DNA sequences generated from the genetic analysis were submitted to Genbank through the BankIt application. This study will support the lack of research on the cephalopod species checklist, particularly in Johor waters. Johor has its marine park called Sultan Iskandar Marine Park (SIMP) in Mersing. 41 islands were grouped into five clusters: Pulau Tinggi, Pulau Sibul, Pulau Besar, Pulau Aur and Pulau Pemanggil (Johor National Parks, 2019). Despite being a marine park, Pulau Tinggi is open for controlled recreational activities for tourism in Johor. Precautionary action and sustainable management plan for cephalopods in Pulau Tinggi should be in place to mitigate any risk of future offence committed by any person.

Materials and Methods

A permit was issued before sampling activities in Pulau Tinggi, Mersing, Johor, Malaysia (Figure 1). This study was conducted under the research permit Prk.ML.630-7 Jld.2 (15) since Pulau Tinggi is a marine park. The sampling took place in the coastal area of Kampung Tanjung Balang, Pulau Tinggi, Johor. Samples were obtained through handpicking during low tide at night and from the local fisherman. The specimens were morphologically identified using the identification keys in FAO Species Catalogue for cephalopods (Jereb & Roper, 2005; Jereb & Roper, 2010; Jereb *et al.*, 2014) and an electronic database World Register of Marine Species for marine species identification at <http://www.marinespecies.org/index.php>. As for the imperfect condition of samples, notes were taken on their features to confirm the species suggested by BLAST analysis. Photographs and measurements of the specimens were taken to assist further in manual identification.

Furthermore, the tissues of specimens were extracted for DNA analysis. DNA analysis started with the extraction of total genomic DNA. The DNA extracts were further processed

through polymerase chain reaction (PCR) using the universal metazoan primers LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer *et al.*, 1994) and the protocols of the PCR kit (exTen 2X PCR Master Mix) with the temperature profile of 94°C in 2 minutes, 94°C in 1 minute of 35 cycles, 48°C in 1 minute, 72°C in 1 minute and finally, followed by extension at 72°C in 5 minutes. After obtaining the bidirectional (forward and reverse) DNA sequencing results from Apical Scientific Sdn. Bhd., the sequences were evaluated using BLAST analysis. Once the identity of the species was confirmed based on the BLAST analysis along with the morphological identification, the sequences were prepared for submission to GenBank. A submission was done through a medium called BankIt, a web-based sequence submission tool.

Results and Discussion

Three species of cephalopod were identified as a result of both morphological identification (Figure 2) and molecular identification (Table 1), which are *Octopus laqueus*, *Uroteuthis*

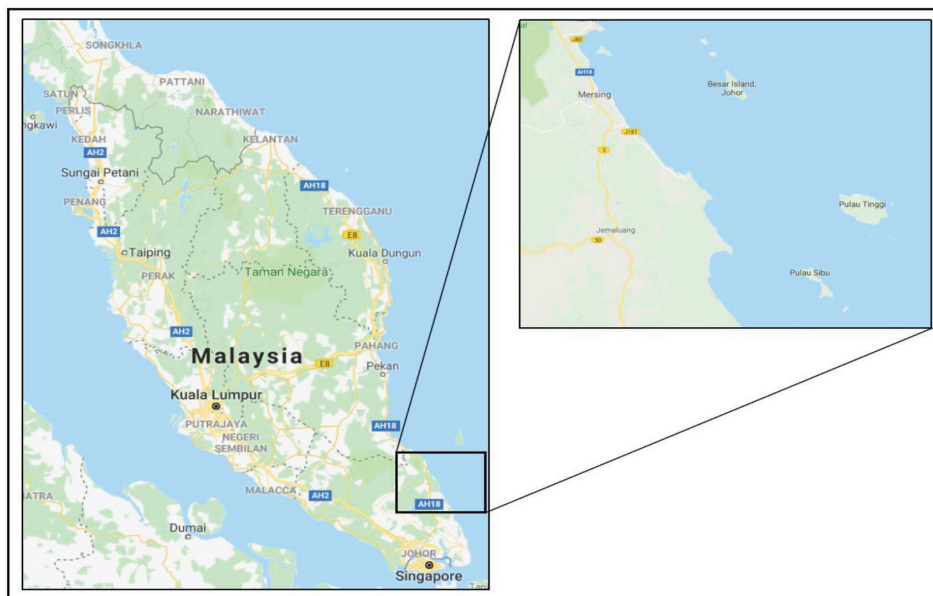


Figure 1: The map of Peninsular Malaysia and the inset map of Pulau Tinggi, Johor
Source: Google (2019)

(*Photololigo*) *chinensis* and *Sepioteuthis lessoniana*. In addition, Table 2 shows the data recorded for each specimen, including the Global Positioning System (GPS) location. Rubaie *et al.* (2012) reported the presence of *Uroteuthis (Photololigo) edulis*, *U. chinensis* and *S. lessoniana* from the family Loliginidae in Sarawak, Malaysia. The COI mitochondrial DNA sequences of the cephalopods from Pulau Tinggi matched with the corresponding sequences in the GenBank database through BLAST analysis. Several studies on animal species identification showed strong resolution level by DNA barcoding (Trivedi *et al.*, 2016)

where the standard COI fragment size was used (658 bp) (Gebhardt & Knebelberger, 2015; Katugin *et al.*, 2015; Lischka *et al.*, 2017). The universal DNA primers of LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') used for the identification of cephalopods showed consistent amplification of COI mitochondrial DNA fragment across a vast array of invertebrates (Folmer *et al.*, 1994). Four partial COI mitochondrial DNA sequences were registered with the GenBank with accession no. MN711655-MN711658 (Table 3).

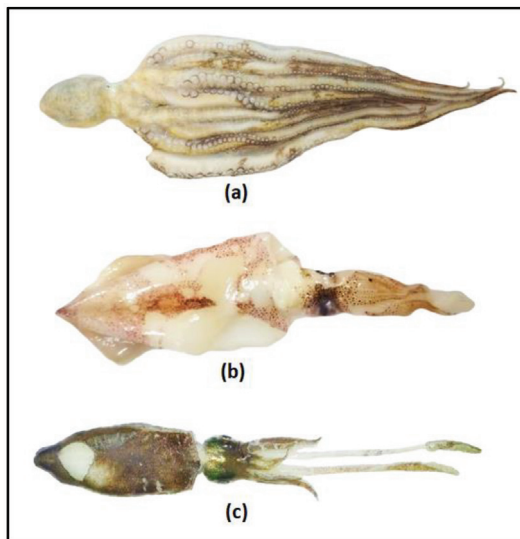


Figure 2: Collected specimens of (a) *Octopus laqueus* (Sample A2), (b) *Uroteuthis (Photololigo) chinensis* (Sample C2) and (c) *Sepioteuthis lessoniana* (Sample B2 and Sample D2) from Pulau Tinggi, Johor, Malaysia. The size of each plate is not following a standard scale

Table 1: Basic Local Alignment Search Tool (BLAST) results

Specimen Sequence	Base Pair (s)	Max Score	Total Score	Query Coverage (%)	E Value	Parent Identity (%)	Accession No. of Corresponding Sequence	Species Identity of Corresponding Sequence
A2_F	683	1133	1133	98	0.0	98.90	AB302176.1	<i>Octopus laqueus</i>
A2_R	683	1125	1125	97	0.0	99.04	AB302176.1	<i>Octopus laqueus</i>
B2_F	686	1074	1074	94	0.0	96.60	KF052434.1	<i>Sepioteuthis lessoniana</i>

B2_R	679	948	948	98	0.0	92.37	LC121571.1	<i>Sepioteuthis lessoniana</i>
C2_F	684	1219	1219	97	0.0	99.70	MG192372.1	<i>Uroteuthis (Photololigo) chinensis</i>
C2_R	679	1170	1170	93	0.0	99.84	MG192372.1	<i>Uroteuthis (Photololigo) chinensis</i>
D2_F	684	1201	1201	95	0.0	99.85	HQ529540.1	<i>Sepioteuthis lessoniana</i>
D2_R	685	1194	1194	95	0.0	99.39	HQ529540.1	<i>Sepioteuthis lessoniana</i>

Table 2: Records of the cephalopod specimens collected in Pulau Tinggi, Johor, Malaysia

No.	Specimen ID	Species	Length (cm)	Weight (g)	Date of Collection	GPS Location
1	A1	<i>Octopus laqueus</i>	24.1	50	14/7/2019	N 2.283978, E 104.117194
2	A2	<i>Octopus laqueus</i>	26.5	46	15/7/2019	N 2.284387, E 104.116074
3	A3	<i>Octopus laqueus</i>	26.5	48	16/7/2019	N 2.285464, E 104.112050
4	A4	<i>Octopus laqueus</i>	34.0	149	30/7/2019	N 2.286070, E 104.111642
5	B2	<i>Sepioteuthis lessoniana</i>	21.1	30	25/7/2019	No record
6	C2	<i>Uroteuthis (Photololigo) chinensis</i>	14.3	14	25/7/2019	No record
7	D2	<i>Sepioteuthis lessoniana</i>	45.0	228	29/8/2019	N 2.311640, E 104.093975

Table 3: The GenBank accession numbers of cephalopods from Pulau Tinggi, Johor, Malaysia

No.	Specimen ID	Species	Accession Number
1	A2	<i>Octopus laqueus</i>	MN711655
2	B2	<i>Sepioteuthis lessoniana</i>	MN711656
3	C2	<i>Uroteuthis (Photololigo) chinensis</i>	MN711657
4	D2	<i>Sepioteuthis lessoniana</i>	MN711658

In addition, the E values of all samples were 0.0, indicating that the nucleotide BLAST search for a match or corresponding sequence was highly significant. Besides that, the query

coverages for all samples were more than 90%, showing that the lengths of the sequences were effective enough to be compared with the sequences in the GenBank. Except for Sample

B2, the other samples had the parent identity in the range of 98.90% to 99.85%, in which it can be concluded that the species status of the samples was the same as the corresponding sequences, i.e., *O. laqueus*, *U. chinensis* and *S. lessoniana*. Sample B2 showed less percentage for parent identity (96.60% for forward reaction and 92.37% for reverse reaction), which could be due to the quality level of the Sample B2 sequences in terms of noise presence. Despite that, the morphological identification (Figure 2) supported the species status of Sample B2 and Sample D2 as *Sepioteuthis lessoniana*. In terms of nucleotide composition, the highest G-C content of 36% was calculated for Sample B2 of *S. lessoniana* and the lowest content of 32% was calculated for Sample A2 of *O. laqueus*. Overall nucleotide composition for all specimens were A (29%), T (35%), C (20%) and G (16%) through mean calculation.

A total of 40 species of cephalopods were recorded in Malaysia by the International Union for Conservation of Nature (IUCN), where 14 of them were in Peninsular Malaysia, 11 in Sabah and 10 in Sarawak (The IUCN Red List of Threatened Species, 2019). The other five might not have enough information in the IUCN database about where the species can be found in Malaysia. For demersal cephalopods in Malaysia, there were five species in total; three of the species were found on the west coast of Peninsular Malaysia, all five species were found on the east coast of Peninsular Malaysia, two of the species were found in Sarawak and only one species found in Sabah (Silvestre *et al.*, 2003). 18 species of cephalopods are being commercialized in Malaysia (Department of Fisheries Malaysia, no date) and in Mersing waters, there were two species recorded of squid and cuttlefish (Muda *et al.*, 2010). Based on Johor Marine Biodiversity Database, there are six species of cephalopods, three of them are an octopus, two are squids and one is cuttlefish in Johor waters (JMarine, 2019).

Cephalopods have been known as one of the protein sources in fisheries for human consumption and due to that, the management of cephalopods is directed mainly towards the

fisheries sector. The exploitation of cephalopods has been well known mostly in southeast Asia, where they have shown the highest in the sale of fished cephalopods through the development of specialized fishing methods such as jigging machines with high-intensity lights (Boyle & Rodhouse, 2005). Their life cycle of rapid growth, high fecundity and short lifespan made them more suited for fisheries exploitation as they can recover rapidly from disturbance and overfishing, thus, they are thought to be resilient (Jereb *et al.*, 2014). However, their life cycle pattern also becomes a problem in assessing fisheries stocks where the usual Maximum Sustainable Yield (MSY) approach applied for other species is unsuitable for cephalopods (Aguilera, 2018). Cephalopods' short lifespan and rapid growth resulted in almost a non-overlapping generation pattern (Boyle & Rodhouse, 2005), where there will be a period of absence of matured cephalopods and the population will be represented by the eggs, their paralarvae and pre-recruits (Arkhipkin *et al.*, 2015). As a result, it is quite difficult to determine the viability of cephalopod stock without a proper understanding of its biology and its interaction with the environment.

Currently, the conservation status of *Octopus laqueus* is not evaluated yet by the IUCN (IUCN Red List of Threatened Species website at <https://www.iucnredlist.org/>), which could be due to the absence of data on the risks that could threaten its population. Evaluation of its population is important for its conservation, hence more studies need to be done. Besides, the conservation status of *Uroteuthis (Photololigo) chinensis* or the mitre squid is evaluated as Data Deficient or unknown by the IUCN, as mitre squid is a commercial species that are fished on a large scale and the catch statistics often did not mention the number of mitre squids caught, thus, assessed how fisheries impact the population of mitre squid complicated (Allcock *et al.*, 2019). The conservation status of *Sepioteuthis lessoniana* is also evaluated as Data Deficient by the IUCN as this bigfin reef squid encounters taxonomic issues and considerable evidence on the morphology and genetics of this species has

shown an exceptionally high degree of cryptic diversity present in this taxon (Cheng *et al.*, 2013).

Conclusion

This study documented three species of cephalopods in Pulau Tinggi, Johor, Malaysia: *Octopus laqueus*, *Sepioteuthis lessoniana* and *Uroteuthis (Photololigo) chinensis*. The species checklist was finalized based on the morphological identification and the Basic Local Alignment Search Tool (BLAST) analysis results of partial protein-coding COI mitochondrial DNA sequences. Four partial DNA sequences of the cephalopod specimens were registered with the GenBank, National Center for Biotechnology Information with accession no. MN711655-MN711658. The conservation status of *Octopus laqueus* is not evaluated yet by the IUCN and the conservation status of *Sepioteuthis lessoniana* and *Uroteuthis (Photololigo) chinensis* are evaluated as Data Deficient. In summary, this study has contributed to the latest update on the species checklist of cephalopod in Pulau Tinggi and the DNA barcodes. The availability of the COI gene sequences of the local cephalopods in the GenBank provides a new resource for future studies on cephalopod species.

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