

DNA barcoding of common catfish in Malaysia

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Abstract. Catfish are widely distributed throughout the tropics around the world and in many countries some species are important source of food, sport fishing and pet. Generally, catfish are found in freshwater area except some species of Ariidae and Plotosidae. Catfish is an important protein source in Malaysia as it being caught vastly throughout the year. However, due to cryptic characteristics expressed by Ariidae specifically, the species identification always lead to mislabelling. Hence, this study identified a total of 31 specimens of 9 ariid species (*Arius leptotacanthus*, *Arius microcephalus*, *Cryptarius truncatus*, *Hemiaris sona*, *Hexanematichthys sagor*, *Nemapteryx nenga*, *Netuma bilineata*, *Netuma thalassina* and *Osteogeneiosus militaris*), and 2 plotosid species (*Paraplotosus albilabris* and *Plotosus lineatus*) found in Malaysia which were run for molecular analysis. Combinations of FishF1 and FishR1 primers were used for the amplification of mitochondrial cytochrome c oxidase I (COI) gene. The generation of Maximum Likelihood with 1000 replicates of the bootstrap using Kimura 2-Parameter method (K2P) showed that all 11 species grouped according to their genera and families. This study also barcoded 9 Ariidae and 2 Plotosidae species found in Malaysia which hopefully will ease the monitoring of the listed species in Malaysia for future study.

Key Words: DNA barcoding, Ariidae, Plotosidae, catfish, Malaysia.

Introduction. Catfish are classified under the order Siluriformes which consist of more than 3000 species from 36 families. Catfish are widely distributed throughout the tropics around the world and in many countries some species are important source of food, sport fishing and pet (Nelson et al 2016). Generally, catfish are found in freshwater area except some species of Ariidae and Plotosidae (Ferraris 2007; Nelson et al 2016). Catfish is an important protein source in Malaysia with Landing Statistics by Department of Fisheries Malaysia (2016, 2017) recorded marine catfish (Ariidae) that have been caught were 19,769 and 19,211 tonnes in 2016 and 2017 respectively. On the other hand, Plotosidae or eel catfish caught in Malaysia have been recorded as 2,550 and 2,409 tonnes in 2016 and 2017 respectively.

Ariidae comprises 153 species from 30 genera with only 26 species under 11 genera recorded in Malaysia (Matsunuma et al 2011; Jalal et al 2012; Yoshida et al 2013; Kimura et al 2015; Froese & Pauly 2019; Fricke et al 2019). Marine ariid catfishes are generally recognizable from other catfish families, both marine and freshwater Siluriformes by several external morphological characteristics. These are namely the number of paired barbels (0 to 3 pairs), presence of a bony plate on their head, stiff and serrated dorsal and pectoral spines, an adipose fin located between the dorsal and caudal fins, and a deeply-forked caudal fin (Kimura et al 2015).

The family Ariidae is the most taxonomic problematic Siluriformes group (Ferraris 2007) due to a high similarity in the species morphology, and a wide distribution throughout the continents but limited specimens in museum collections (Marceniuk & Menezes 2007). Although many studies have been done to define and to reclassify ariids (Sullivan et al 2006; Marceniuk & Menezes 2007; Betancur-R 2009; Marceniuk et al 2012; Aguilera & Marceniuk 2018), the species identification based on morphology merely still can be confusing (Yu & Quilang 2014). The application of molecular

identification is also crucial in support of the species identification based on morphology (Betancur-R 2009; Marceniuk et al 2012). As such, the introduction of DNA barcoding system has helped to resolve species identification by using short and standardized gene regions as species tags (Hebert & Gregory 2005). DNA barcoding is an effective method to overcome species misidentification that is solely done by morphological observation (Hubert et al 2015; Abdullah et al 2017).

Plotosidae is another Siluriformes family which contains marine water species. There are 10 genera (*Anodontiglanis*, *Cnidoglanis*, *Euristhmus*, *Neosiluroides*, *Neosilurus*, *Oloplotosus*, *Paraplotosus*, *Plotosus*, *Porochilus*, and *Tandanus*) with about 40 species in the world (Allen & Feinberg 1998; Ng & Sparks 2003; Nelson et al 2016). Plotosidae are widely distributed throughout freshwater, brackish water and marine water of the Indo-West Pacific region and extends to Japan (Ferraris 2007; Yoshino & Kishimoto 2008). Four species in Malaysia under 2 genera were recorded (*Paraplotosus* and *Plotosus*) that are *Paraplotosus albilabris* (Valenciennes, 1840), *Plotosus abbreviatus* Boulenger, 1895, *Plotosus canius* Hamilton, 1822, *Plotosus lineatus* (Thunberg, 1787). Generally, Plotosidae are distinguished from other Siluriformes due to the presence of 4 pairs of barbel, dendritic organ, and caudal fin confluent with dorsal procurrent caudal and anal fin (eel-like tail).

This study is important as the studies on species status of Siluriformes (Ariidae and Plotosidae) in Malaysia are limited. The study also implemented the DNA barcoding method of Siluriformes (Ariidae & Plotosidae) in Malaysia. This study also describes the phylogenetic relationship of the common catfish found in Malaysia. This study developed DNA barcodes for 12 commonly found catfish species in Malaysia, namely: *Arius leptanotacanthus* Bleeker 1849, *Arius microcephalus* Bleeker 1855, *Cryptarius truncatus* (Valenciennes 1840), *Hemiaris sona* (Hamilton 1822), *Hexanematichthys sagor* (Hamilton 1822), *Nemapteryx nenga* (Hamilton 1822), *Netuma bilineata* (Valenciennes 1840), *N. thalassina* (Rüppell 1837), *Osteogeneiosus militaris* (Linnaeus 1758), *Paraplotosus albilabris* (Valenciennes, 1840) and *Plotosus lineatus* (Thunberg, 1787). An updated checklist of the common catfish of families Ariidae and Plotosidae found in Malaysia is presented, with potentially cryptic species highlighted based on DNA evidence.

Material and Method

Sample collection and identification. A total of 31 specimens of 11 commercially and economically important catfish species in Malaysia were collected from February 2016 until January 2017. These included *A. leptanotacanthus* (n = 2), *A. microcephalus* (n = 2), *C. truncatus* (n = 4), *H. sona* (n = 1), *H. sagor* (n = 2), *N. nenga* (n = 4), *N. bilineata* (n = 2), *N. thalassina* (n = 1), *O. militaris* (n = 3), *P. albilabris* (n = 3) and *P. lineatus* (n = 5). The specimens were initially identified based on their morphologies. Kailola (2004), Marceniuk & Menezes (2007) and Ng (2012) were used for the identification of Ariidae; *A. leptanotacanthus*, *A. microcephalus*, *C. truncatus*, *H. sona*, *H. sagor*, *N. nenga*, *N. bilineata*, *N. thalassina* and *O. militaris*. Ferraris (1999) was referred for the identification of *P. albilabris* and *P. lineatus*. Each specimen was photographed on the dorsal and the left side views using Nikon D90 SLR camera. A piece of white muscle tissue approximately 30 mg was excised from the right side of the body of each specimen. The tissue was placed in a 1.5-mL microcentrifuge tube containing absolute ethanol and stored at room temperature until processed for the DNA.

DNA extraction and PCR amplification. Approximately 20 mg of muscle tissue was subjected to DNA extraction using Promega Wizard Genomic DNA purification kit (Madison, WI) following the manufacturer's protocol. Combinations of the following forward (FishF1) and reverse (FishR1) primers were used for the amplification of approximately 655 bp of the mitochondrial cytochrome c oxidase I (COI) gene (Ward et al 2005):

FishF1 [5'-TCAACCAACCACAAAGACATTGGCAC-3']
FishR1 [5'-TAGACTTCTGGGTGGCCAAAGAATCA-3']

Polymerase chain reactions (PCR) were done in 20 µL volumes. The PCR mix consisted of 12 µL exTEN 2x PCR Mastermix (1st BASE), 1.0 µL of each primer (0.01 mM), 5 µL of ultrapure water and 1.0 µL of DNA template. The PCR conditions were as Ward et al (2005) with little modifications; initialization for 3 min at 95°C followed by 34 cycles of denaturation for 30 sec at 95°C, annealing for 40 sec at 52°C, and extension for 1 min at 72°C. A final extension step at 72°C for 5 min completed the reaction. Around 20 µL PCR products were loaded for each well of 1.7% agarose gels (1 × TBE) with MIDORIGreen Advance (Nippon Genetics Europe GmbH), and visualized. Approximately PCR products 650 bp sized bands were excised from the gel. The bands were cut and sent to Apical Scientific Sdn. Bhd. (previously 1st BASE Sdn. Bhd.) in Selangor Darul Ehsan, Malaysia for purification and sequencing.

DNA sequence analysis. The consensus COI sequences were obtained and aligned using MEGA6 software (Tamura et al 2013). Additional sequences were downloaded from BOLD (Barcode of Life Data Systems, www.boldsystems.org) and GenBank for validation and for some analyses (Table 2). Pairwise genetic distances within species, genus and family and between species were calculated using the Kimura 2-Parameter method (K2P) (Kimura 1980). The phylogenetic tree were analysed by running the Neighbor-Joining (NJ) and Maximum Likelihood (ML) trees with 1000 replicates of the bootstrap test.

Both ML and NJ trees at 1,000 pseudoreplicates (Saitou & Nei 1987) were constructed using the K2P model. K2P genetic distances and NJ trees were produced using the MEGA version 6 software (Tamura et al 2013). COI sequences were also submitted to GenBank, with accession numbers MK604248–MK604252, and MN094539–MN094554.

Results and Discussion

Phylogenetic tree. The present study generated phylogenetic tree based on Maximum Likelihood (ML) and Neighbor-Joining (NJ) trees at 1,000 replicates using Kimura 2-Parameter (K2P). Both trees generated by ML and NJ showed similar topology hence only tree generated by ML is discussed in this paper. Additionally, the best fit DNA test were run using MEGA6 (Tamura et al 2013) and showed that ML is the best phylogenetic tree that fit this study which fits the 24 different nucleotide substitution models. Models with the lowest BIC (Bayesian Information Criterion) scores are considered to describe the best substitution pattern. For estimating ML values, a tree topology was automatically computed involving 34 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 474 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al 2013).

The tree generated showed that all 11 species grouped according to their genera and families (Figure 1). Eight gene sequences were extracted from GenBank; *H. sona* (KU894611), *H. sagor* (JX198211), *N. thalassina* (HQ149893), *O. militaris* (KY849538), *P. albilabris* (KF604676) and *P. lineatus* (JN313096). The outgroup sequences for the tree are also Siluriformes members, *Pangasius pangasius* of Pangasidae and *Mystus singaringan* of Bagridae.

A. leptonotacanthus, *A. microcephalus*, *C. truncatus*, *H. sona*, *H. sagor*, *N. nenga*, *N. bilineata*, *N. thalassina* and *O. militaris* were clustered together with 100% bootstrap while *P. albilabris* and *P. lineatus* were clustered together with 92% bootstrap.

COI sequences of *P. pangasius* and *M. singaringan* were extracted from GenBank with accession no. KM232630 and KU692662, respectively. The outgroups were clustered together with 57% bootstrap.

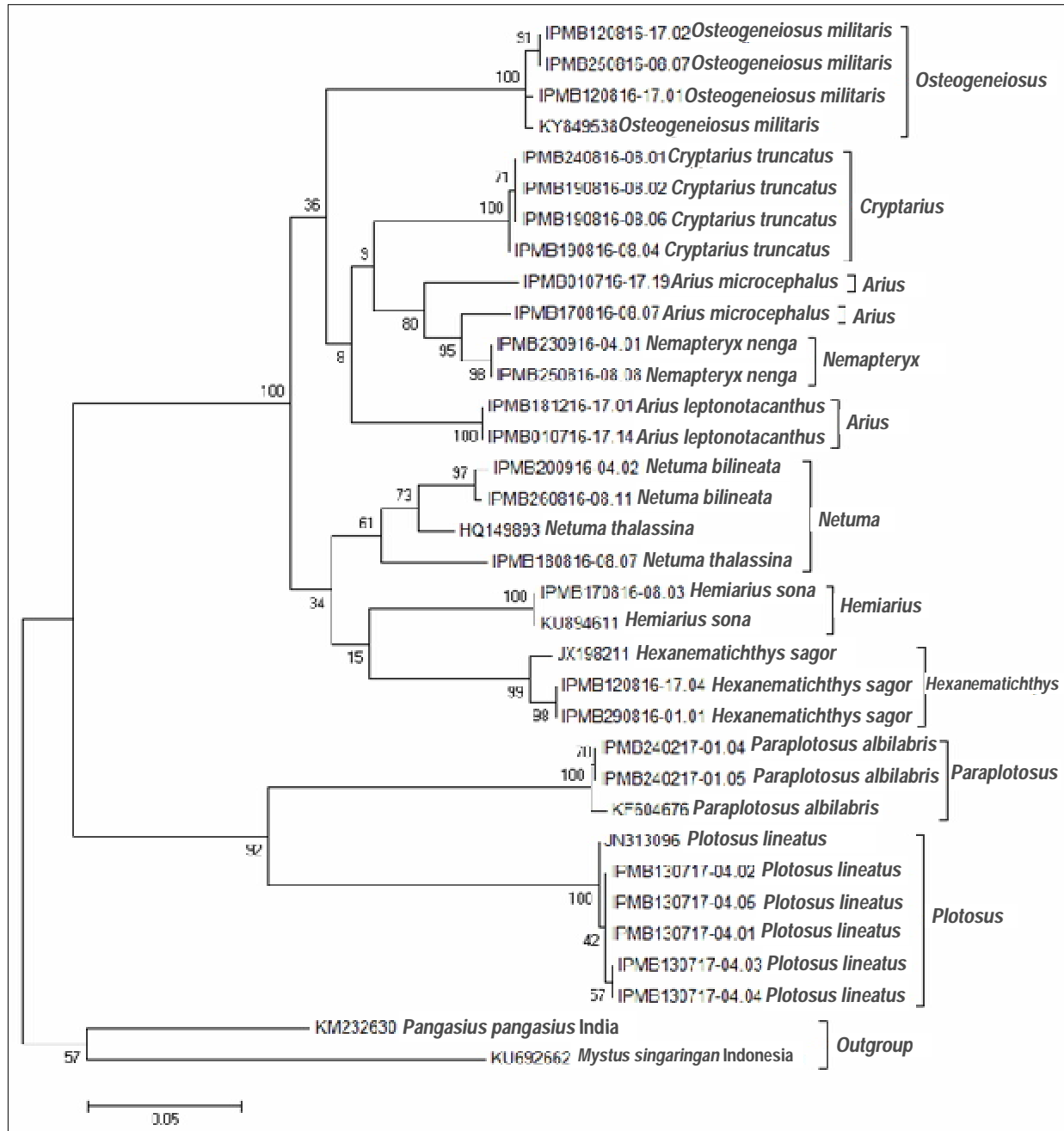


Figure 1. Phylogenetic tree based on Maximum Likelihood (ML) at 1,000 replicates using Kimura 2-Parameter (K2P) of common catfish in Malaysia.

Ariidae analysed in this study consisted of 9 species (*A. leptotacanthus*, *A. microcephalus*, *C. truncatus*, *H. sona*, *H. sagor*, *N. nenga*, *N. bilineata*, *N. thalassina* and *O. militaris*). The species are grouped into two clades; *Hemiaris*, *Hexanematichthys* and *Netuma* in one clade while other Ariinae are grouped in another clade *Netuma*, *Hemiaris* and *Hexanematichthys* are subcladed together with 34% bootstrap support. The clade formed support the study by Marceniuk et al (2012) that formed *Netuma* and *Hexanematichthys* in the same subclade. The clade of other Ariinae consists of *A. leptotacanthus*, *A. microcephalus*, *C. truncatus*, *H. sona*, *N. nenga* and *O. militaris* with 36% bootstrap support which supports the previous study by Betancur-R (2009). The ML phylogenetic tree which form other Ariinae of the present study formed upper clade with 41% bootstrap supports the ML tree in study by Betancur-R (2009).

Previous studies on DNA barcoding of Plotosidae in Malaysia mostly focus on *Plotosus canius* (Khalili Samani et al 2016). However, instead of *Plotosus canius*, this study managed to record *P. albilabris* and *P. lineatus*. *P. lineatus* and *P. albilabris* formed a monophyletic cluster. The clade of Plotosidae in Figure 1 shows that *P. albilabris* is sister to *P. lineatus* with 92% bootstrap support.

Genetic distance. Tables 1-4 show the genetic distance within and between species for both families (Ariidae and Plotosidae) and Table 5 shows the pairwise distance of all species that was calculated based on Kimura-2 parameter (K2P). The intraspecific distance within Ariidae was calculated as 0.091 (± 0.008) while the intraspecific distance within Plotosidae is calculated as 0.125 (± 0.01). The outgroups are referred to the *P. pangasius* and *M. singaringan*. Genetic distance increased with increasing taxonomic levels, supporting the idea of a significant change in genetic divergence (Hebert et al 2003). The genetic distance within groups of Ariidae ranged between 0.000 (± 0.000) and 0.064 (± 0.09) while the genetic distance between genera of Ariidae ranged between 0.059 (± 0.009) and 0.144 (± 0.020). The genetic distances within groups of Ariidae and between genera of Ariidae are similar to the previous study by Abdullah et al (2017). The study reported the genetic distance within species of Ariidae ranged between 0.000 and 0.042, while the genetic distance between species of Ariidae ranged between 0.004 and 0.169.

The closest genetic distance between genera of Ariidae can be observed between *Arius* and *Nemapteryx*. *Nemapteryx* in this study refers to *N. nenga* which was previously placed under *Arius* (Marceniuk & Menezes 2007). However, the placement of *N. nenga* under *Nemapteryx* followed study by Kailola (2004) which then being accepted as the valid name (Ferraris 2007; Kottelat 2013). The most distant Ariidae can be seen is between *Osteogeneiosus* and *Hexanematichthys* with the genetic distance of 0.144 (± 0.020). *Osteogeneiosus* was described as the basal unit of Ariidae based on osteological and other morphological characters by Jayaram & Dhanze (1986).

The genetic distance between genera of Plotosidae were 0.206 (± 0.021). This can be correlated with the genetic distance in the previous study of *P. canius* and *P. lineatus* which recorded the range between 0.231 and 0.252 (Khalili Samani et al 2016). Although *P. albilabris* and *P. lineatus* represented two different genera, *Paraplotosus* and *Plotosus*, the genetic distance of the two species are quite similar to the genetic distance found in the previous study by Khalili Samani et al (2016). Plotosidae (eel catfishes) is a monophyletic taxa (Oliveira et al 2001; Sullivan et al 2006). Generally, plotosids, the eeltail catfishes are characterised with the dendritic organ near anus, the venomous pectoral and dorsal spines, and also the confluent dorsal, caudal and anal fins. Plotosidae occur in both marine and brackish water in the Indian Ocean and Indo-West Pacific (Ferraris 2007; Nelson et al 2016).

Ariidae is known for their problematic taxonomy due to their cryptic characteristics. The highly similar morphology of Ariidae always leads to mislabelling of the species, even for taxonomists. Molecular analysis has shown a clearer taxa of Ariidae as well as Plotosidae, which are similar to the previous studies (Marceniuk & Menezes 2007; Betancur-R 2009; Marceniuk et al 2012; Khalili Samani et al 2016). The Barcoding of Life (BOLD) Initiative is an effort to store all of DNA barcodes in the same library. This effort is more efficient and useful with the standard genetic marker used to tag each species (Ekrem et al 2007). The present study has barcoded 9 Ariidae species (*A. leptonotacanthus*, *A. microcephalus*, *C. truncatus*, *H. sona*, *H. sagor*, *N. nenga*, *N. bilineata*, *N. thalassina* and *O. militaris*) and 2 Plotosidae species (*P. albilabris* and *P. lineatus*) found in Malaysia waters. Ariidae and Plotosidae can be found abundantly in brackish and coastal water of Malaysia.

Table 1

Genetic distance between genera of Ariidae

<i>Species</i>	<i>Arius</i> (<i>n</i> = 4)	<i>Cryptarius</i> (<i>n</i> = 4)	<i>Hemiarius</i> (<i>n</i> = 2)	<i>Hexanematichthys</i> (<i>n</i> = 3)	<i>Nemapteryx</i> (<i>n</i> = 2)	<i>Netuma</i> (<i>n</i> = 4)	<i>Osteogeneiosus</i> (<i>n</i> = 4)
<i>Cryptarius</i>	0.092						
<i>Hemiarius</i>	0.098	0.107					
<i>Hexanematichthys</i>	0.117	0.120	0.113				
<i>Nemapteryx</i>	0.059	0.072	0.092	0.097			
<i>Netuma</i>	0.097	0.106	0.094	0.106	0.093		
<i>Osteogeneiosus</i>	0.113	0.106	0.140	0.144	0.116	0.134	
<i>Outgroup</i>	0.219	0.207	0.235	0.228	0.223	0.194	0.225

Table 5

Pairwise distance of Ariidae and Plotosidae

<i>Species</i>	<i>Arius</i> (<i>n</i> = 4)	<i>Cryptarius</i> (<i>n</i> = 4)	<i>Hemiarius</i> (<i>n</i> = 2)	<i>Hexanematichthys</i> (<i>n</i> = 3)	<i>Nemapteryx</i> (<i>n</i> = 2)	<i>Netuma</i> (<i>n</i> = 4)	<i>Osteogeneiosus</i> (<i>n</i> = 4)	<i>Paraplotosus</i> (<i>n</i> = 3)	<i>Plotosus</i> (<i>n</i> = 6)
<i>Cryptarius</i>	0.092								
<i>Hemiarius</i>	0.098	0.107							
<i>Hexanematichthys</i>	0.117	0.120	0.113						
<i>Nemapteryx</i>	0.059	0.072	0.092	0.097					
<i>Netuma</i>	0.097	0.106	0.094	0.106	0.093				
<i>Osteogeneiosus</i>	0.113	0.106	0.140	0.144	0.116	0.134			
<i>Paraplotosus</i>	0.232	0.229	0.237	0.237	0.233	0.222	0.231		
<i>Plotosus</i>	0.230	0.234	0.226	0.249	0.235	0.238	0.262	0.205	
<i>Outgroup</i>	0.219	0.207	0.235	0.228	0.223	0.194	0.225	0.231	0.238

Table 2

Genetic distance within genera of Ariidae

<i>Genus</i>	<i>d</i>	<i>SE</i>
<i>Arius</i>	0.064	0.009
<i>Cryptarius</i>	0.001	0.001
<i>Hemiaris</i>	0.000	0.000
<i>Hexanematichthys</i>	0.010	0.003
<i>Nemapteryx</i>	0.000	0.000
<i>Netuma</i>	0.044	0.007
<i>Osteogeneiosus</i>	0.005	0.002
<i>Outgroup</i>	0.194	0.022

Note: The table shows the genetic distance, *d* and the standard error, *SE* for each genus of Ariidae.

Table 3

Genetic distance within groups of Plotosidae

<i>Genus</i>	<i>d</i>	<i>SE</i>
<i>Paraplotosus</i>	0.003	0.002
<i>Plotosus</i>	0.001	0.001
<i>Outgroup</i>	0.181	0.019

Note: The table shows the genetic distance, *d* and the standard error, *SE* for each genus of Plotosidae.

Table 4

Genetic distance between genera of Plotosidae

<i>Species</i>	<i>Paraplotosus (n = 3)</i>	<i>Plotosus (n = 6)</i>
<i>Plotosus</i>	0.206	
<i>Outgroup</i>	0.220	0.234

Conclusions. This study describes the phylogenetic relationship of the common catfish found in Malaysia. This study showed that the genetic distance ranges of Ariidae species are smaller as compared to the genetic distance range of Plotosidae species. The low genetic distance can relate to the high similarity of a family. This can hence relate to the cryptic characteristics of ariids, while plotosids can be easily identified. Therefore, the implementation of DNA barcoding in identifying a species especially the ariids is an efficient approach to discriminate and identify fish species. On the other hand, this study updates the species status of Ariidae and Plotosidae found in Malaysia. This study identified 9 Ariidae species and 2 Plotosidae species. Hopefully, this study will ease the monitoring of the listed species in Malaysia for future study as well as for the sustainable management of the Malaysian fisheries.

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