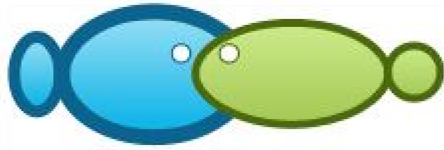

Morphological and molecular identification of mullet species (Mugilidae) from Setiu Wetland, Terengganu, Malaysia

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Abstract. Mullet (Mugilidae) or known as belanak are common fishes in Malaysia. However, only four genera (5 species) of mullet were recorded in Terengganu so far. The objective of the study was to identify the mullet specimens from Setiu Wetlands, Terengganu using morphological and molecular perspectives to produce a reliable result. Hence, both approaches were applied to accurately identify the mullet to the species level. Morphological identification was done by measuring the morphometric and meristic characters of the specimens and to compare it with the Mugilidae taxonomic keys. The result for morphometric measurements and meristic counts obtained, matched the similarities described by previous authors as *Moolgarda perusii*. Further analysis was made to access the utility of partial cytochrome c oxidase subunit 1 (*CO1*) gene to delineate the mullet specimens. PCR amplification was conducted using the universal primers and approximately 660-bp of partial *CO1* gene was sequenced for all specimens. Molecular identification was performed by deploying the specimens' partial *CO1* gene in the GenBank using the basic local alignment search tool (BLAST). BLAST results showed that the specimens were resolved with high similarity as *M. perusii* from the GenBank. Next, to investigate the evolutionary relationship of the specimens with other relevant species of the same family, phylogenetic trees using Neighbour-Joining, Maximum-Likelihood and Maximum-Parsimony algorithms were constructed. The results indicated that the mullet specimens collected from Setiu Wetland, Terengganu were confirmed as *M. perusii*. Hence, the applications of both morphological and molecular approaches are useful to successfully determine the mullet species.

Key Words: Mugilidae, Setiu Wetland, morphological measurement, genetic identification, cytochrome c oxidase subunit 1 (*CO1*).

Introduction. Mullet are great food fishes and are probably the most widely distributed economical fishery in the world's coastal waters (Nash & Shehadeh 1980). Mullet are generally distributed in tropical, temperate, brackish waters and some live in freshwater (Thomson 1984; Nelson 2006). As of 2017, there are 75 valid species within the order Mugiliformes in the world (Thomson 1997; Eschmeyer & Fong 2017). Several studies such as Mansor et al (1998), Ambak et al (2010), Matsunuma et al (2011) and Fishbase website (Froese & Pauly 2012) have documented the description and information of mullet species in Malaysia. The systematic relationships of this group in Malaysian waters have been updated and subdivided into ten genera of which are *Cestraeus*, *Crenimugil*, *Chelon*, *Liza*, *Ellochelon*, *Moolgarda*, *Mugil*, *Paramugil*, *Oedalechilus* and *Vagamugil*. The Department of Fisheries Malaysia had recorded that 6,149 tonnes of mullet (belanak) were landed in year 2016 (Department of Fisheries Malaysia 2016). Most of the landings were recorded in northwestern part of Peninsular Malaysia and also in Sabah and Sarawak. However, commercial mullet landing have not been recorded in Terengganu. Thus, the identification of the mullet species in Setiu Wetland, Terengganu is essential for fisheries management. In addition, there is no report of genetic assessment in

authenticating the species of the mullet in Terengganu. Nevertheless, genetic evidence will greatly assist in the unambiguous identification and classification of living organisms to the species level. Hebert et al (2003) suggested the use of the mitochondrial DNA gene cytochrome oxidase subunit 1 (*CO1*) as a global bio-identification system for animals. Considering the effectiveness of *CO1* in molecular identification, initiatives such as The Barcode of Life Database (http://www.barcodinglife.org) and The Fish Barcode of Life (http://www.fishbol.org) incorporated a DNA-based identification system that is based on the *CO1* gene.

Concerning the phylogeny of the Mugilidae family, it appears exceptionally obscure at both the intra- and interspecific levels as it is extremely challenging to distinguish among species (Papasotiropoulos et al 2002). The effectiveness of *CO1* gene in mitochondrial DNA has been promising in distinguishing morphologically cryptic organisms including fishes (Ward et al 2005; Khan et al 2010; Kamaruzzaman et al 2011; Arjunaidi et al 2016). Difficulties with animal groups owing to highly overlapping morphological characters that are of taxonomic value have been experienced before (Ghajarieh et al 2006; Blasco-Costa et al 2008; Liu & Zhao 2010; Li et al 2011). Hence, the implementation of both approaches, molecular assessment and morphological identification, is a vital step towards precise and accurate identification. As such, FAO (2013) recommended the integration of genetic evidence for healthy and sustainable fisheries management.

This study will provide some information on the differentiation of taxonomic units based on morphometric and meristic characteristics in mullet specimens found in Setiu Wetland besides authenticating its phylogenetic status. The information is crucial in establishing a genetic database that will assist in the conservation and management of the vast diversity of Malaysian fisheries stocks. Also, precise identification will lead to understanding the pattern of distribution. Thus, a well-managed fisheries resource will benefit economically and prevent the occurrence of low genetic diversity populations. Hence, the objective of this study is to determine the identification of the mullet using the approaches of morphological assessment and using *CO1* gene analysis.

Material and Method. Ten mullet specimens from Setiu Wetlands, Terengganu, Malaysia were obtained from South China Sea Repository and Reference Centre, Institute of Oceanography and Environment (RRC, INOS), Universiti Malaysia Terengganu. The specimens were caught with the use of seine net by local fishermen in 2014. Fish muscle and fins were cut using dissecting scissors and were fully immersed in 95% ethanol and kept at -20°C.

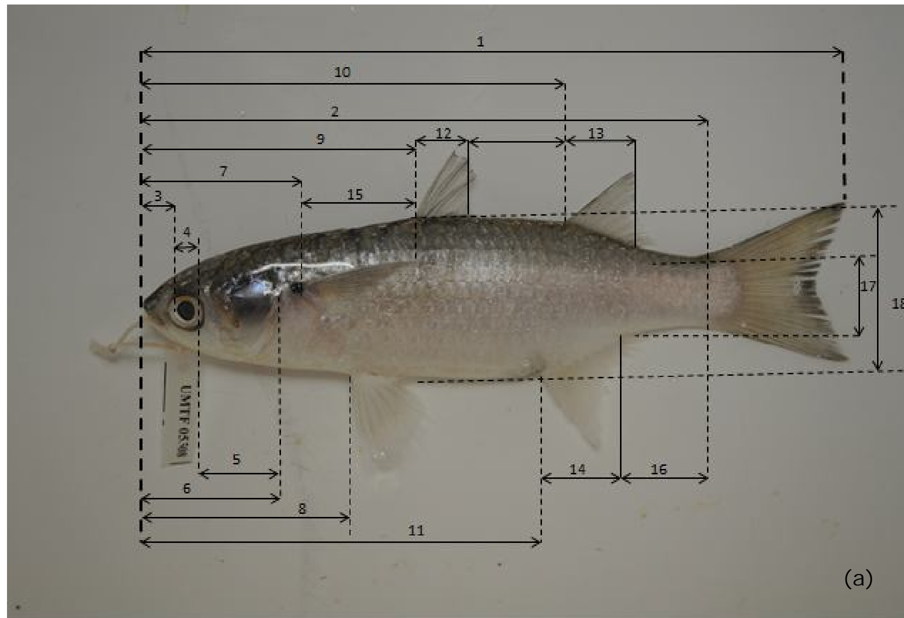
Morphological assessment. Morphological features of the Mugilidae specimens were compared following the keys and descriptions by Harrison & Senou (1997). The morphometric measurements were done with a digital vernier caliper to the nearest 0.01 milimeter (mm) and the meristic characters were counted. All measurements and characters were measured and counted three times for each specimen. Species identification was completed based on their meristic counts and morphometric characteristics. A total of 34 morphometric measurements (Tables 1 and 2, and Figure 1) and 12 meristic counts were taken for each specimen.

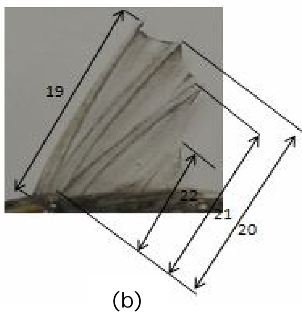
The twelve meristic characters counted: first dorsal fin spines (DF 1), second dorsal fin ray (DF 2), anal fin spine (AF 1), anal fin ray (AF 2), caudal fin ray (CF), pectoral fin ray (PF 1), pelvic fin ray (PF 2), scales in longitudinal series till origin of pectoral fin (LSPF), scales in longitudinal series till origin of second dorsal fin (LSDF 2), scales in longitudinal series till origin of caudal peduncle (LSCP), scales in transverse series around caudal peduncle (TSCP), scales in transverse series from origin of 1st dorsal fin (TSDF 1).

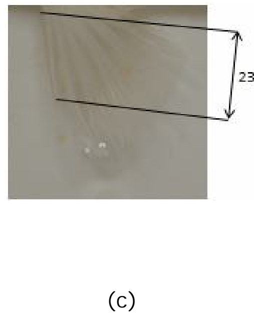
Table 1

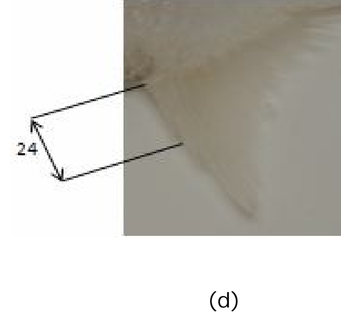
Morphometric measurement and meristic count used in this study

Number	*Description*	*Acronym*
1	Total length	TL
2	Standard length	SL
3	Snout length	ML
4	Eye diameter	ED
5	Post orbital length	EOD
6	Head length	HL
7	Pre-pectoral length	PrPcD
8	Pre-pelvic length	PrPeD
9	Pre-dorsal length	PrDD 1
10	Second pre-dorsal length	PrDD 2
11	Pre-anal length	PrAD
12	First dorsal fin base	DL 1
13	Second dorsal fin base	DL 2
14	Anal fin base	AL
15	Pectoral fin length	PcL
16	Caudal peduncle length	PeL
17	Caudal peduncle depth	CPDe
18	Body depth of origin at first dorsal fin	BDe
19	First dorsal spine length	DSL 1
20	Second dorsal spine length	DSL 2
21	Third dorsal spine length	DSL 3
22	Fourth dorsal spine length	DSL 4
23	Pelvic spine length	PeSL
24	First anal spine length	ASL 1
25	Pectoral fin insertion-dorsal fin origin	PcDD 1
26	Pectoral fin insertion-second dorsal fin origin	PcDD 2
27	Pelvic fin insertion-dorsal fin origin	PeDD 1
28	Pelvic fin insertion-second dorsal fin origin	PeDD 2
29	Anal fin origin-dorsal fin origin	ADD 1
30	Anal fin origin-second dorsal fin origin	ADD 2
31	Pelvic fin insertion-anal fin origin	PeAD
32	Pectoral fin insertion-anal fin origin	PcAD
33	Pectoral fin insertion-pelvic fin insertion	PcPeD
34	First dorsal fin origin-second dorsal fin origin	D1D2D

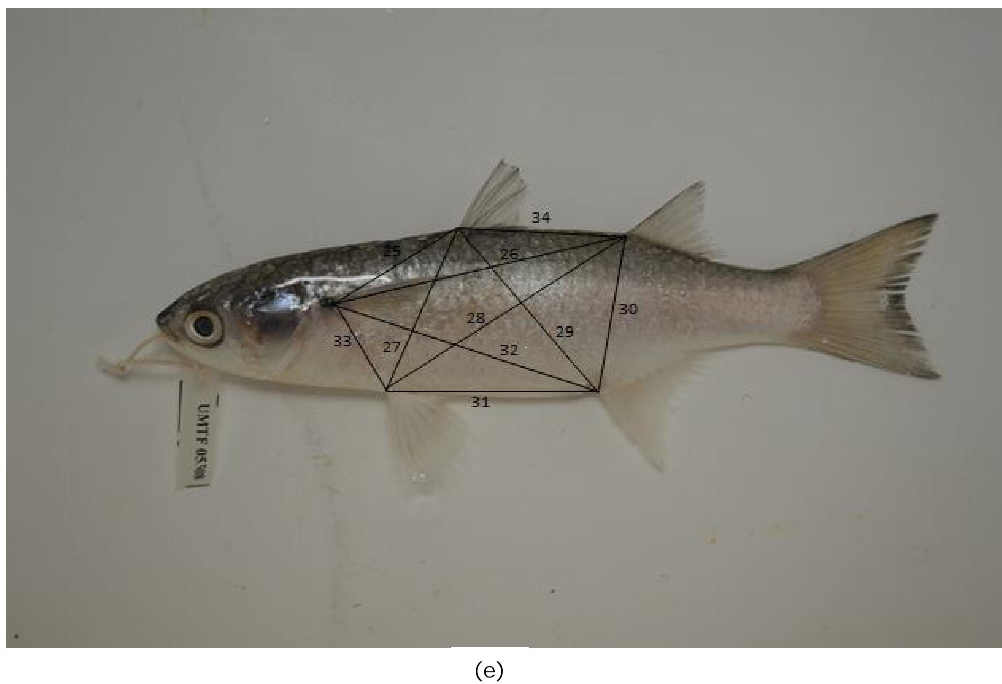








(b) (c) (d)



(e)

Figure 1. Morphometric measurement of the mullet specimens (a)-(e). The numbers indicate the characters as described in Table 1.

Table 2

Morphometric measurement of mullet

Number	*Character*	*Mean±SD*	*Range*
1	TL	190.8±32.0	176.00-240.00
2	SL	146.6±10.0	138.00-158.00
3	ML	9.67±2.9	6.89-12.58
4	ED	5.71±2.6	4.17-9.45
5	EOD	21.18±4.1	17.74-26.02
6	HL	34.46±4.7	31.76-41.18
7	PrPcD	38.64±4.6	34.33-43.58
8	PrPeD	55.11±7.5	48.09-63.02
9	PrDD 1	70.41±17.2	45.01-79.33
10	PrDD 2	107.59±7.6	101.72-116.88
11	PrAD	103.48±10.5	94.3-115.25
12	DL 1	16.64±3.8	12.15-19.77
13	DL 2	17.14±2.1	14.88-19.1
14	AL	20.74±2.0	18.74-22.77
15	PcL	33.03±2.4	30.37-35.08
16	PeL	24.78±2.3	22.51-27.14
17	CPDe	17.43±3.2	16.2-22.61
18	BDe	37.48±3.0	33.41-39.54
19	DSL 1	20.31±2.0	18.15-22.19
20	DSL 2	19.67±2.4	16.84-21.66
21	DSL 3	17.6±2.4	14.85-19.71
22	DSL 4	9.3±1.8	7.72-11.34
23	PeSL	16.00±1.1	15.09-17.34
24	ASL 1	8.28±2.0	5.84-9.86
25	PcDD 1	38.41±15.9	14.44-46.27
26	PcDD 2	72.21±6.4	65.31-78.14
27	PeDD 1	40.70±5.8	34.67-46.28
28	Pe DD 2	65.33±4.4	61.11-69.86
29	ADD 1	49.95±4.5	45.90-54.93
30	ADD 2	35.26±4.3	31.86-40.40
31	PeAD	52.61±4.4	49.57-58.32
32	PcAD	72.68±16.4	65.03-97.72
33	PcPeD	28.63±5.3	21.81-32.34
34	D1D2D	36.31±5.4	30.79-41.48

Molecular analysis. The total genomic DNA was extracted using the Cell/Tissue DNA Extraction Kit (Spin-column) (BioTeke Corporation, China). The polymerase chain reaction (PCR) was conducted to amplify the partial fragment of cytochrome c oxidase subunit 1 (*CO1*) gene. Amplification of the *CO1* gene fragments was carried out using the universal primers: FishF1 (5′-TCA ACC AAC CAC AAA GAC ATT GGC AC-3′) and FishR1 (5′-TAG ACT TCT GGG TGG CCA AAG AAT CA-3′) (Ward et al 2005).

PCR was carried out in 25 μL reaction mixture containing 2.5 μL of 10× PCR Buffer, 2 μL of DNA template, 1 μL of each primer (25 mM), 2 μL of dNTPs (2.5 mM) and 0.3 μL of Taq polymerase. The PCR cycling conditions consisted of an initial 5 minutes of denaturation at 95°C followed by 35 cycles for each of the reactions; 40 seconds at 94°C, 40 seconds at 48°C and 1 min at 72°C. Lastly, an elongation step for 7 minutes at 72°C completed the cycle (Bowen et al 2008). The amplifications were done in a Thermal Cycler (Eppendorf AG, Germany).

Prior to sequencing, the PCR products were purified using the DNA Purification Kit (BioTeke Corporation, China) according to the manufacturer's instructions. All the purified PCR products were sent to First Base Laboratories Sdn. Bhd. and sequenced bi-directionally using the same primers as those for the PCR.

Genetic analysis. The raw sequences of the specimens were edited using eBioX (http://www.ebioinformatics.org/ebiox/). The chromatograms were viewed using 4Peaks (Nucleobytes Inc.) and FinchTV (Geospiza Inc.) as references to identify any noises and gaps. Unwanted sequences were then removed. All of the sequences were identified using the nucleotide Basic Local Alignment Search Tool (BLAST) (Altschul et al 1990) at the National Center of Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). As a general rule, a top match with a sequence similarity of at least 98% was used as a criterion to designate potential species identifications (Barbuto et al 2010).

All sequences were then aligned together using CLUSTAL-W program in MEGA software (version 6.06) (Tamura et al 2011). Several Mugilidae species were used as sister group (*Moolgarda perusii*, Assession number: JQ060505.1; *Moolgarda cunnesius*, Assession number: JQ045777.1; *Liza tade*, Assession number: EU014263.1; *Moolgarda engeli*, Assession number: JQ431911.1; *Moolgarda seheli*, Assession number: JQ045781.1; *Valamugil robustus*, Assession number: JF494777.1). In addition, *Lates calcarifer* (Assession number: FJ384689.1) was used as an out-group. Also, MEGA version 6.06 was also used to construct phylogenetic trees using Neighbour-Joining (NJ) algorithm, Maximum-Likelihood (ML) algorithm and Maximum-Parsimony (MP) algorithm. Kimura-2 parameter was chosen as best-fit model. The genetic distance was also calculated using MEGA version 6.06. The confidence level of the trees was tested with 1000 replications (Tamura et al 2011).

Results and Discussion. The morphologies of mullet specimens from Setiu Wetlands, Terengganu were examined to determine its species by referring to Harrison & Senou (1997), Mansor et al (1998), Ambak et al (2010) and Matsunuma et al (2011). The specimens possessed a rather elongated second dorsal and anal fin and its caudal fin is emarginated. The specimens' thoracic and abdominal scales were observed to be more distinctly ctenoid. Phenotypically, the specimens' body were greenish colour at its dorsal, the flanks and the colour of the abdomen is silvery. In addition, there was dark spot at the origin of the pectoral fins. All these appearances were in concurrent with that of *Moolgarda perusii* stated by Matsunuma et al (2011). The mullet specimens in the present study were also observed to have an average BDe of 25.56% of SL. Harrison & Senou (1997) advocated that *M. perusii* BDe from SL ranged from 24 to 35%. In addition, the PcL of the present study was 22.53% of SL, which also corresponds to the description by Harrison & Senou (1997).

Based on the meristic counts of rays, there were some features of the mullet specimens from Setiu Wetland that were congruent with that of *M. perusii* described by Matsunuma et al (2011), who stated that the meristic characters of *M. perusii* were D IV + 8-10; A III, 9; P_1 15-17; LR 31-34. However, the meristic counts of mullet specimens in the present study were D IV + 8-9; A III, 8-10; PF 1 or P_1 13-15; PF 2 4-6; LR 31-37 (LR labeled as LSCP in the present study). The specimens possessed lower account of soft rays for pectoral fin. Also, the longitudinal scales until the base of the caudal fin (labeled as LR: Matsunuma et al 2011; labeled as LSCP: present study) were higher than that described by Matsunuma et al (2011). Despite that, the LSPF 8-11, LSDF2 19-21, TSCP 14-17 and TSDF1 11 of the mullet specimens are congruent with the description by Harrison & Senou (1997). Hence, the collected morphological data of the mullet specimens collected from Setiu Wetland, Terengganu were similar to *M. perusii*.

Genetic analysis. A sequence of 660-base-pair of partial *CO1* gene was amplified for all the mullet specimens through PCR amplification. The *CO1* sequences were deposited in GenBank under accession number KY130504.1-KY130513.1. BLAST analysis revealed that all specimens were resolved as *M. perusii* with high similarity (99-100%). In

addition, pairwise distance showed that all specimens shared high genetic similarity ranging from 99.4-100% with *M. perusii* (available in GenBank). Further investigation through phylogenetic relationship reveals that the mullet specimens collected from Setiu Wetland formed a monophyletic clade with *M. perusii* (Figure 2). All specimens were separated from the sister group and the out-group. Thus, this showed species-specificity of the specimens confined within the species *M. perusii*. However, a single specimen (Mu-SW 03) was separated from the group.

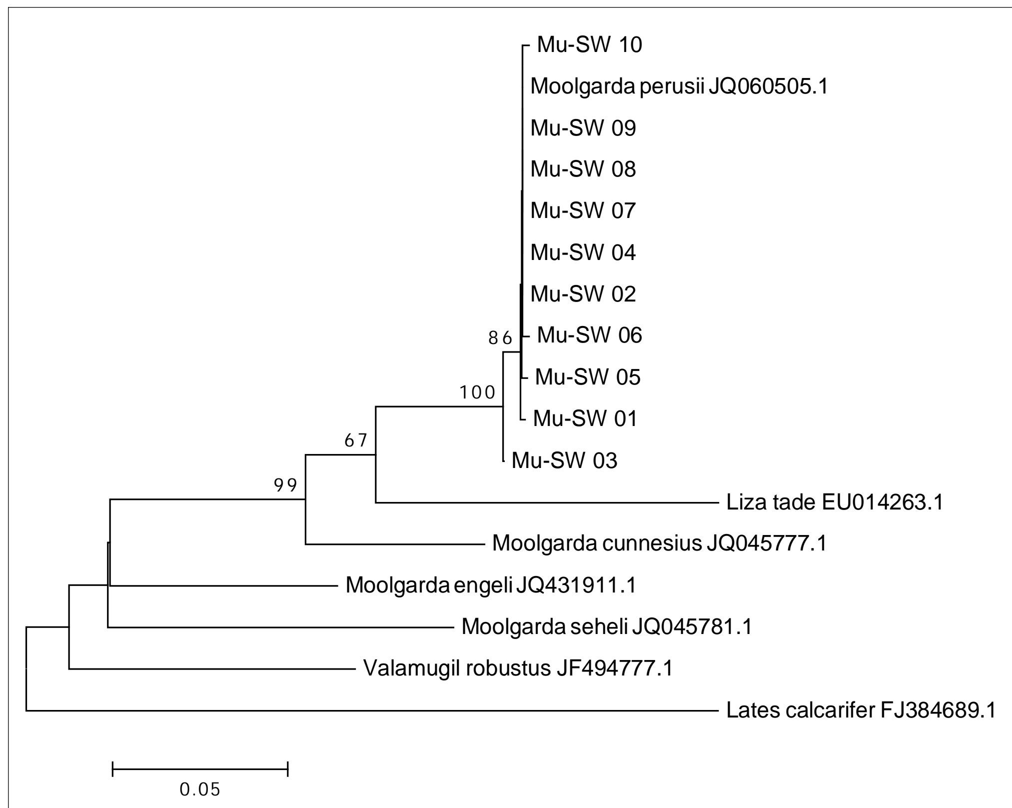


Figure 2. Phylogenetic tree of mullets collected from Setiu Wetland, Terengganu constructed with Neighbour-Joining algorithm and Kimura-2 parameter as best-fit model. Confidence level was set to 1,000 replications. The value at each node is the bootstrap probability value. Sister groups and out-group were named, followed with the Genbank accession numbers, respectively. Specimens were labeled with abbreviations Mu-SW (mullet Setiu Wetland).

Analysis using the Maximum-Parsimony and Maximum-Likelihood algorithms also showed similar patterns. The single specimen (Mu-SW 03) was then further analyzed by deploying to BOLDSYSTEM (http://www.boldsystems.org/). The results showed that the specimen was resolved as *M. perusii* with 99.49% similarity. Hence, this showed that all the specimens were confirmed as *M. perusii*. Department of Fisheries Malaysia (2016) reported that there are no landings of mullets in Terengganu. However, mullet is a common fish that can be found in the Northern regions of Peninsular Malaysia. Most of the species studied were *Liza subviridis* (Chen 1977; Fatema et al 2013). To date, there are no literatures that incorporated the use of both morphological and molecular identification of mullets in Malaysia. Application of *CO1* marker has been revealed to be an essential tool in discriminating the species of mullets as well as resolving the mullet taxonomic ambiguity (Kumar et al 2011; Rahman et al 2013). In addition, *CO1* marker has also been applied in studies regarding genetic diversity, phylogeography, phylogenetic and evolutionary lineage (Shen et al 2011; Sun et al 2012; Polyakova et al 2013).

The combination of both morphometric and molecular approaches using *CO1* marker has authenticated the species of mullets found in Setiu Wetland. Phylogenetic analysis showed that it is evident the species is *M. perusii*. Further study should be done

by incorporating other genetic markers, both of mitochondrial and nuclear genes. Coyle (1998) suggested that stock identification must be done thoroughly by combining several methods from both genetic analysis and phenotypic approaches. Morever, Durand et al (2012) had successfully recognized the 20 genera of Mugilidae through the application of multiple genes.

Conclusions. The mullets of Setiu Wetland were confirmed as *M. perusii* based on the identification of both morphological and molecular approaches. The morphological characters of the mullet specimens in this study is congruent with those that has been described by previous studies. The identification of this species is important as it could be added into the vast and intricate taxonomic status of Mugilidae family. Moreover, it serves as a basis of stock identification for fishery management of Malaysian stocks.

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