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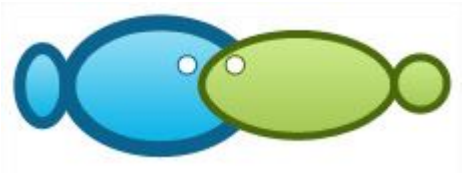

Authentication of *Tenualosa* species in Perak River, Malaysia: application of morphological measurement and molecular analysis of partial *CO1* and 16S genes to resolve species ambiguity

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Abstract. Malaysia possessed two species of *Tenualosa* sp.; the *Tenualosa toli* and *Tenualosa macrura*, which can only be found in Sarawak. Recently, samples that resembled the *Tenualosa* sp. were found in Perak River of Peninsular Malaysia. The absence of comprehensive study of *Tenualosa* sp. in Peninsular Malaysia had urged this study to be conducted to authenticate the samples found. Identification were done using morphological analysis with the integration of molecular assessment using partial genes of cytochrome c oxidase subunit 1 (*CO1*) and 16S rRNA. Morphological analysis of 40 samples showed the closest correlation with *Tenualosa ilisha* (Hamilton, 1822). However, variations were found in meristic counts and the samples were differentiated into two groups. The meristic counts also overlapped with other species of the same genus. Molecular assessments on 10 samples performed using partial *CO1* and 16S rRNA genes had obtained 500-bp and 573-bp sequences respectively, that has high similarity with *T. ilisha*. Further genetic assessment through phylogenetic analysis showed that all 10 samples formed a monophyletic lineage with *T. ilisha*. However, the samples were significantly separated with others of the same species from different countries. The findings suggested that there is a possibility the samples might migrate from other countries and had experienced domestic speciation towards the local environment. The findings of this study would be useful in recognizing the potential fisheries resources. This will greatly help in planning and implementing a healthy and sustainable exploitation of this species.
Key Words: *Tenualosa* sp., morphological assessments, cytochrome C oxidase subunit 1 gene, 16S rRNA gene.

Introduction. *Tenualosa* species or known as Terubok is an important and high commercial value clupeid fish. There are five species of *Tenualosa* spp. can be found worldwide and two species are common in Malaysia. These two species are *Tenualosa toli* and *Tenualosa macrura* which can be found only in Sarawak waters (Blaber et al 1996; Phillip 2001). The Sarawak *Tenualosa* species is one of the most significant estuarine fish that is considered as one of the Malaysian commercial fish species that contributes to the national economy. In Peninsular Malaysia, catching of *Tenualosa* species is rare and inconsistent in the past few decades (Gambang 1988). It has been presumed that the *Tenualosa* species has subsequently extinct, however no such records has ever been confirmed. Recently, catch landings of *Tenualosa* species in Peninsular Malaysia has been proclaimed by several local fishermen in Perak River. The reappearance of *Tenualosa* species in Perak River has known to be dissappeared for almost 20 years has raised several research questions such as its distribution, taxonomy, and systematics. However, *Tenualosa* species in Perak River has never received enough attention, hence

no comprehensive studies are done. Consequently, there is lack of information regarding the *Tenualosa* species in Perak River. To date, this species is still unidentified and there is no current study to officially validate its species even though the *Tenualosa* species in Perak bare resemblance with the *Tenualosa* species in Sarawak. Thus, the current status of *Tenualosa* species in Perak River is still poorly understood.

As the identification of this species that are caught in Perak River remains unclear, this might lead to the difficulty of collecting any information regarding the species. Insufficient knowledge and information may leave the *Tenualosa* species in Perak unattended without any efforts of its preservation and conservation management in Perak River. Pollution and overfishing might occur and could lead to unsustainable exploitation and the declination in numbers of this species that might render its extinction. Such incidents have been observed in the other two species; the *T. toli* and *T. macrura* in Sarawak as the numbers have been reported drastically decreasing in the past few years (Phillip 2001; Rahim et al 2014). Abdul Aziz et al (2015) reported that genetic diversity of *T. toli* was relatively low for two populations in Sarawak and might cause a genetic deprivation towards its population. Therefore, as an initial step, identifying the species in Perak River will acknowledge the local community and authorities regarding the value of the species and efforts to manage the species can be implemented. Moreover, *Tenualosa* species in Perak River can become an alternative source of Malaysian *Tenualosa* species supply besides Sarawak. Herein, it is hypothesized that the *Tenualosa* species in Perak River is of different species from the *T. toli* and *T. macrura* from Sarawak.

One of the best ways to precisely identify species is through morphometric study accompanied by molecular identification. Kartavtsev et al (2006) reported that molecular approach is more accurate in species identification compared to morphological character identification alone. Several studies have been done through the application of both approaches to precisely validate and identify the aquatic species (Watanabe et al 2008; Kolangi-Miandare et al 2013; Haniffa et al 2014). The development of species-specific morphological and molecular markers has been done to successfully monitor natural population stocks (Tinti et al 2003). It has been demonstrated that both markers can be applied to investigate the species divergence and genetic variation within geographical regions, hence relatively mapping the distribution (Motamedi et al 2014). As a result, efforts to manage natural fisheries population stocks can be implemented and applied to maintain sustainability. Such studies have been done in natural populations of *Rastrelliger* spp. and *Channa striata* in Malaysia (Darlina et al 2011; Song et al 2013). However, as any information regarding the *Tenualosa* species in Perak, identification has been poorly documented, the efforts to manage the natural populations cannot be done. This might cause unsustainable exploitations of fisheries resources and once again causing the dissappearance of valuable *Tenualosa* species resources in the future. Therefore this study was conducted to identify the *Tenualosa* species in Perak through morphological and molecular approaches.

Material and Method. Samples of *Tenualosa* species were collected from the Perak River in Bagan Datoh, Perak from January - March 2014. Samples were caught using drift net by the fishermen. A total of 40 samples were collected and used for the experiment. White muscle tissue and fin clips were collected and preserved in 95% ethanol and stored at -20^{o}C for further genetic analysis. The tissue and clips were taken on the right side of the samples to allow the left side to be used for morphological analysis.

Morphological analysis. The samples were identified morphologically according to the verification of Narejo et al (2008), Munroe et al (1999) and Hong et al (2013). Twenty five morphometric characters were measured using digimatic digital caliper (Mitutoyo, Japan) with an accurancy of 0.01 mm and seven meristic characters were counted using standard methods. The procedure of fixation, photograph and preservation for the specimen are followed Seah et al (2011). Methods of counting and measuring are generally followed Hubbs & Lagler (1964) and terminology of morphological features and descriptions are modified from Seah et al (2009). Measurements of the morphometric

characters were standardized in order to eliminate any size effect: Standardized measurement = Morphometric Parameter / Standard Length*100.

DNA extraction, Polymerase Chain Reaction (PCR) amplification and sequencing. Total genomic DNA of 10 samples were extracted using DNA extraction kits, the Cell/Tissue DNA Extraction Kit (Spin Column) (BioTeke Corporation, China). Two molecular markers were used, the partial cytochrome c oxidase subunit 1 (*CO1*) and 16S gene marker.

The *CO1* gene was amplified using two primers based on Jannatul-Farihah et al (2011) which were CO1f (5'-CCTGCAGGAGGAGGAGAYCC-3') and CO1r (5'-CCAGAGATTAGACCGAAATCAGTG-3'). A PCR mixture containing 2.5 µL of 10× PCR buffer; 0.8 µL of 25 mM $MgCl_2$; 0.6 µL of 100 pmol of both primers; 0.4 µL of 5U Taq DNA polymerase (Vivantis, Malaysia); 2.4 µL of 10,000 uM dNTP; and 3.0 µL of 100 ng DNA sample was prepared in a 25 µL reaction. The cycling profile for *CO1* began with the initial step of 3 minutes at 94°C, followed by 30 cycles of denaturation (at 94°C for 30 seconds), annealing (at 52°C for 40 seconds) and extension (at 72°C for 60 seconds) and finally subjected to the final extension (at 72°C for 10 minutes).

The 16S gene was amplified using primers 16SarL (5'- CGCCTGTTTATCAAAAACAT -3') and 16SbrH (5'- CCGGTCTGAACTCAGATCACG T-3') (Palumbi 1996). A PCR reaction with a total volume of 25 µL were prepared using 2.5 µL of 10× PCR buffer (readily added with $MgCl_2$); 0.3 µL of 10 µM of both primers; 0.2 µL of 5U Taq DNA polymerase (TransGen Biotech Co., Ltd, China); 0.25 µL of 10,000 uM dNTP; and 4.0 µL of 100 ng DNA sample. PCR cycling conditions used were as follow: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 93°C for 30 s, primer annealing at 55°C for 40 s and chain elongation at 72°C for 60 s, follow by final extension at 72°C for 5 min. Both the amplifications were done in a Thermal Cycler (Eppendorf AG, Germany).

Prior to sequencing, the PCR products were purified using a DNA purification kit (BioTeke Corporation, China) according to the manufacturer instruction. All the purified amplicons were then sequenced bi-directionally by First Base Laboratories Sdn. Bhd. using the same primers for PCR amplification.

Sequence alignment and evolutionary analysis. A Macintosh-based software; eBioX (http://www.ebioinformatics.org/ebiox/) were used to edit the obtained sequences. The chromatograms were analyzed and inspected for nucleotides conformity using the software 4Peaks (Nucleobytes Inc.) and FinchTV (Geospiza Inc.). The processes were to remove any unwanted sequences, noise, gaps and stop codons to precisely validate the sequences. The sequences were then identified using 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). Next, the edited sequences were aligned together with sequences of other *Tenualosa* species available in the GenBank (*T. toli*, Accession number: AP011600.1; *T. thibaudeaui*, Accession number: AP011604.1). *T. ilisha* from other countries such as from India, Thailand and West Africa were also included (*T. ilisha* Thailand, Accession number: AP011611.1; *T. ilisha* India 1, Accession number: AP011610.1; *T. ilisha* India 2, Accession number: NC016682.1; *T. ilisha* India 3, Accession number: DQ400344.1; *T. ilisha* West Africa, Accession number: EU552785.1). This is to assist in inferring the possibility of evolutionary origin of the samples found in Perak River.

The alignment was performed in MEGA software through CLUSTAL W program (version 5.2) (Tamura et al 2011). *Lutjanus argentimaculatus* (Accession number: DQ400344.1) and *Cromileptes altivelis* (Accession number: KC845547.1) were used as outgroup. Phylogenetic trees of Neighbour-Joining, Maximum-Parsimony and Maximum-Likelihood were constructed to resolve the evolutionary lineage amongst the *Tenualosa* species. A best-fit DNA substitution model of Kimura-2-parameter was used in NJ and ML analyses with the confidence level of 1000 replications (Tamura et al 2011).

Results and Discussion. Morphometric and meristic analysis of *Tenualosa* species collected from Perak River revealed that more than 90% of the samples had been identified as *T. ilisha*. Out of 25 morphometric and seven meristic characters, five morphometric and four meristic characters were chosen to identify the samples collected

significantly. Morphological assessments showed that the samples could be differentiated into two groups based on the variations of morphometric measurement and meristic count (Table 1). The average total length and standard length ± S.D for group A were 30.6±2.59 and 24.3±1.78 respectively whereas for group B were 30.2±1.71 and 22.8±1.36 respectively. Narejo et al (2008) made a comparison between two types of *T. ilisha* by grouping and differentiated the morphometric and meristic characters based on size range. The result of Total Length (TL), Standard Length (SL) and Fork Length (FL) and meristic of the study had been used to compare with the results of present study to assist in identifying the *Tenualosa* species in Perak River.

Table 1

Comparison of *Tenualosa ilisha* morphological characters between the two groups (Group A and Group B) and *Tenualosa ilisha* from Pakistan (Type A and Type B)

	Tenualosa sp. (Group A)		*Tenualosa sp.* (Group B)		*Tenualosa ilisha (Narejo et al 2008)*			
					Type A		*Type B*	
Morphometric	*Min-Max*	*Mean ± S.D.*	*Min-Max*	*Mean ± S. D.*	*Length groups* 25.1-30.0*	*Length groups* 30.1-35.0*	*Length groups* 25.1-30.0*	*Length groups* 30.1-35.0*
					Mean ± S.D.	*Mean ± S.D.*	*Mean ± S.D.*	*Mean ± S.D.*
Total length (TL) (cm)	27.4-34.8	30.6± 2.59	27.4-34.1	30.2± 1.71	28.0± 1.90	32.1± 2.10	27.0± 1.55	33.4± 1.55
Standard length (SL) (cm)	22.4-27.2	24.3± 1.78	20.0-25.3	22.8± 1.36	22.2± 1.33	26.0± 1.88	21.5± 1.20	27.0± 1.55
Fork length (FL) (cm)	25.0-30.0	26.9± 1.97	20.8-28.6	25.6± 2.02	23.5± 1.88	27.5± 1.33	23.0± 1.22	28.5± 1.15
Head length (HL) (cm)	6.4-7.8	6.9± 0.43	5.0-7.4	6.5± 0.49	6.1± 0.80	7.3± 0.65	5.3± 0.60	5.5± 0.33
(HL as % to SL)	26.6-31.0	28.6± 1.09	20.2-32.4	28.7± 1.93	-	-	-	-
Eye diameter (ED) (cm)	1.0-1.5	1.2± 0.13	1.0-1.5	1.2± 0.11	1.1± 0.55	1.2± 0.44	1.0± 0.25	1.0± 0.22
(ED as % to SL)	4.3-5.5	5.0± 0.41	4.6-6.4	5.3± 0.45	-	-	-	-
Meristic	*Range*	*Mean ± S.D.*	*Range*	*Mean ± S.D.*	*Range*	*Mean ± S.D.*	*Range*	*Mean ± S.D.*
Dorsal fin rays	18-20	-	16-18	17±1.0	17-19	18±1.0	18-20	19±1.0
Pectoral fin rays	14-15	-	12-15	13±1.0	15-16	14±1.0	14-15	13±1.0
Pelvic fin rays	8	-	7-9	8±0.0	7-9	8±1.0	8-10	9±1.0
Anal fin rays	19-21	-	15-20	18±1.0	18-22	20±2.0	19-23	21±1.0

*Length groups (cm) of samples used, taken from Narejo et al (2008).

Results of the present study showed that the HL as percentage of SL for group A and group B were 28.6% and 28.7%, respectively. Whitehead (1985) reported that the HL for *T. ilisha* is 28 to 32% of its SL, for *T. macrura* is 22 to 25% of SL, for *T. reevesii* is 27 to 33% of SL, for *T. thibaudeaui* is 30 to 33% of SL and *T. toli* is 25 to 27% of SL. However, as results showed are overlapping to more than one species of *Tenualosa*, the *Tenualosa* species from Perak cannot be accurately identified. Nevertheless, the range of meristic characters of both groups was quite similar to Narejo et al (2008). Narejo et al (2008) found that the *T. ilisha* in Pakistan have 17-20 dorsal fin rays (DFR), 14-16 pectoral fin rays (PCFR), 7-10 pelvic fin rays (PVFR), 18-23 anal fin rays (AFR). As in this study, the Group A showed the DFR was 18-20, PCFR was 14-15, PVFR was 8 and the AFR was 19-21. However, *Tenualosa* species from Perak - group B has meristic characters that are slightly out of range and not in consensus with Narejo et al (2008). Group B showed the DFR was 17±1.0, PCFR was 13±1.0 and AFR was 18±1.0, these are less than those from Group A and Pakistan *T. ilisha*. Arai & Amalina (2014) recorded the presence of *T. ilisha* in Malaysian waters by examining fourteen samples using morphological characters only. The results of the study reported that the *T. ilisha* has a measurement HL of 26.5-30.0%, which concurrent with the present study. However, for the meristic characters, Arai & Amalina (2014) reported that the DFR is 17-18, PCFR is 13-15, PVFR is 8, and AFR is 18-21, which is quite variable than the result of the present study. Forty samples are

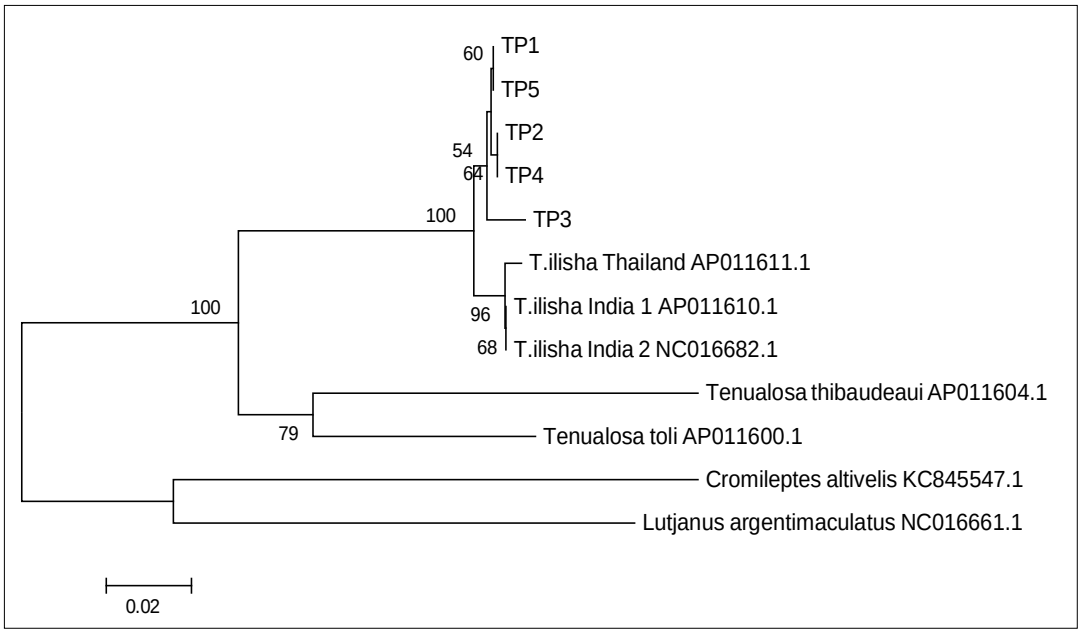
examined in the present study and the morphological characters are ambiguous. Thus, the results indicate that there are morphological variations amongst the *Tenualosa* species in Perak. Variations of morphological characters of the specimens might be related to the point of origin and the aquatic ecosystems they thrived in. Study by Narejo et al (2008) emphasized that the length-weight relationship of *T. ilisha* was found to be significantly different in accordance to the winter-summer seasons.

Variations in morphological characters have also been observed in populations of *Puntius bimaculatus* in terms of physio-chemical parameters within different altitudinal range of river basin (De Silva & Liyanage 2009). Furthermore, morphological variations amongst marine fish species have been documented in Baltic Sea herring (*Clupea harengus*) (Jorgensen et al 2008) and bluefish (*Pomatomus saltatrix*) (Turan et al 2006). Both studies were done by examining samples throughout oceanic system with no physical barriers, thus suggesting the possibility of occurrence of migration. Turan et al (2006) suggested that variations in morphometric and meristic characters might also be influenced by several factors such as different feeding environment, prey types and food availability due to different environmental factors. Turan et al (2006) also exerted that differentiation in phenotypic traits do not tend to hinder gene flow between populations but also implied that the fish groups might not mix extensively. Present study indicates that there is a possibility that Group A of *Tenualosa* species in Perak might migrate from the Indian Ocean whereas Group B might be a later generation of *T. ilisha* of Group A. However, further studies should be done to confirm this matter.

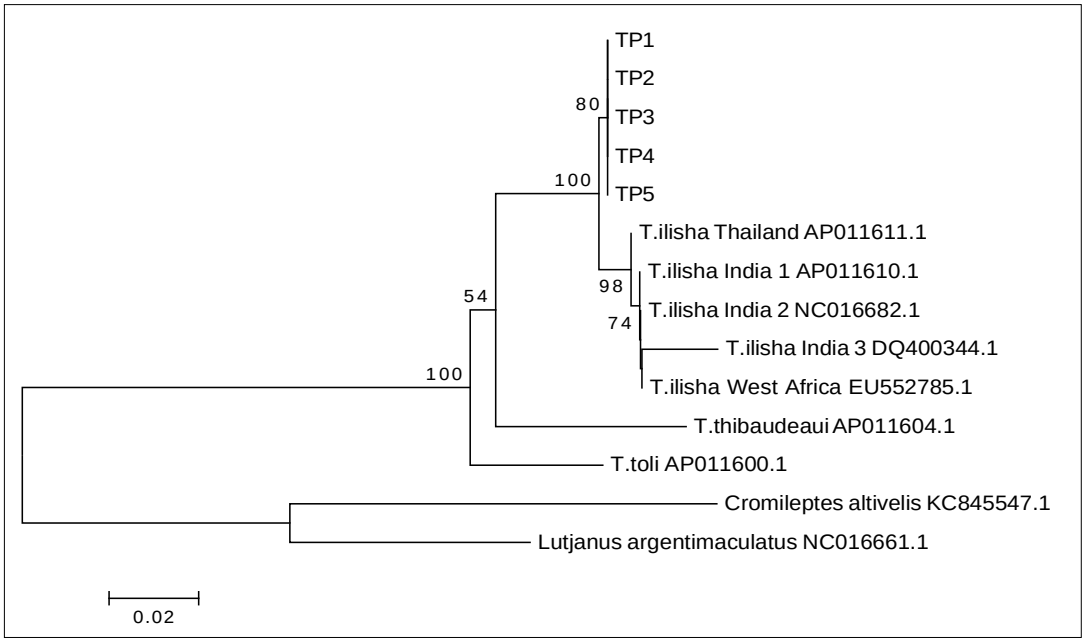
The variations of morphological characters in *Tenualosa* species in Perak has led to the used of molecular tools to genetically identify the samples. Two molecular markers of *CO1* and 16S rRNA has been employed to determine the identity of *Tenualosa* species found in Perak River. Sequence analysis of the amplified fragments of partial *CO1* and 16S revealed a sequence of 500-base-pair and 573-base-pair, respectively for each samples. The *CO1* and 16S sequences were deposited in GenBank under accession numbers KP128692-KP128696 (*CO1*) and KP128687-KP128691 (16S). BLAST results showed that all samples had 99% similarity with mitochondrial DNA *CO1* and 16S sequences of *T. ilisha* (Accession Number: AP011611.1) in the GenBank Database, authenticating that all the samples are *T. ilisha*. The low genetic distance (*CO1*: < 0.017, 16S rRNA: < 0.028) between *Tenualosa* species in Perak and other *T. ilisha*, further support the species authentication. Phylogenetic tree of Neighbour-Joining (NJ), Minimum-Evolution (ME), Maximum-Parsimony (MP) and Maximum-Likelihood (ML) showed similar patterns in which all samples of *Tenualosa* species in Perak were separated from the sister group and outgroup and resolved within the same monophyletic clade with other *T. ilisha*.

Interestingly, all samples of *Tenualosa* species in Perak formed a single distinctive clade that is independent from other lineage of *T. ilisha* from Thailand, India and West Africa (Figure 1). Both genes separated the *Tenualosa* species in Perak with strong statistical support (100%). This suggested that there is a possibility the *Tenualosa* species in Perak is a distinctive and isolated species of *T. ilisha* that can be differentiated from other fishes of the same species. In addition, the evolutionary divergence of *Tenualosa* species in Perak from its monophyly lineage might also indicate that they also share a common ancestor. A comprehensive study should be done in the future involving a wider geographical range and higher number of samples. By doing so, investigation of genetic structuring might confirm the hypothesis. Therefore, the origin and evolutionary history of Tenualosa species in Perak could be possibly determined. Such case has been documented on the Telmatherinidae fishes (Stelbrink et al 2014). Study done on *Sphaeramia orbicularis* demonstrated the occurrence of genetic differentiation in populations of isolated fishes of the same species (Hanzawa et al 2012). Each population will have its own unique haplotypes. However, a common haplotype shared between several populations indicates the possibility of a closer inter-population relationship through a common ancestor in which has been documented in studies done on *Feneropenaeus chinensis* (Li et al 2009), *Channa striata* (Siti-Balkhis et al 2011), *Odontesthes* spp. (Garcia et al 2014) and *Tenualosa toli* (Abdul Aziz et al 2015). Plath et al (2010) in their study on Atlantic molly (*Poecilia mexicana*), suggested that speciation

might occur without the limitation of geographical barriers and through domestic adaptation to different environment.



(a)



(b)

Figure 1. Phylogenetic position of *Tenualosa* sp. from Perak River of Peninsular Malaysia compared to Thailand, India and West Africa *CO1* (a) and 16S rRNA (b) gene sequences. Neighbour-Joining tree constructed using Neighbour-Joining algorithm with Kimura-2 parameter modal. Value at each node is the bootstrap value (1000 replicate). Genbank accession numbers are provided after the species name. *Cromileptes altivelis* (Serranidae) and *Lutjanus argentimaculatus* (Lutjanidae) are used as outgroup. Specimens in the presence study are labeled with the initial TP (*Tenualosa* species in Perak).

Further study revealed that local adaptation towards varied environment greatly influences the molecular characteristics (Kelley et al 2012). *T. ilisha* has been reported to have great capability of long distance migration (up to 700-1200km) (Pillay & Rosa 1963; McDowall 1988). Hence, there is a possibility of migration and local adaptation of *T. ilisha* of the Indian Ocean through several different countries as there is limitless connectivity of the oceanic systems. The results of genetic assessment are congruent with the

morphological analysis. In contrary to study done by Arai & Amalina (2014), meristic counts investigated in forty samples of *Tenualosa* species found in Perak River exhibits slight variation from those of *T. ilisha.* This might possibly correlate with the evolutionary lineage that showed significant separation of *Tenualosa* species found in Perak River with the other *T. ilisha*. The findings of the present study would assist in acknowledging the existence of *T. ilisha* in Malaysia. This would help in designing and implementing conservation programs in managing these fisheries resources in the future.

Conclusions. The present study has successfully identified *Tenualosa* species in Perak of Peninsular Malaysia, which has been described to be extinct for the past few decades. Through the means of both morphometric and molecular approaches, the species of *Tenualosa* species in Perak has been identified as *T. ilisha*. This is the first recorded study to morphologically and genetically authenticate the *Tenualosa* species in Perak in Malaysia. Though, this study has found that there are variations in morphometric and meristic characters. The morphological variations suggest the possibility that the samples are divided into two groups with the second group is the later localized generation of the first group. In addition, the genetic relationship of Malaysia *T. ilisha* is closest to that of Thailand, followed with the Indian and West Africa. These raise other research questions. Therefore, further study should be done to understand more about the biology and life history of Malaysian *T. ilisha* that inter-related with its genetic characteristics. Such studies are important to prevent this invaluable species from disappearing again in the future.

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