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Validation of the occurrence of the tropical eels, *Anguilla bengalensis bengalensis* and *A. bicolor bicolor* at Langkawi Island in Peninsular Malaysia, Malaysia

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Abstract: The anguillid eels found in Peninsular Malaysia were identified, using morphological analysis as *Anguilla bengalensis bengalensis* and *A. bicolor bicolor* and that identification was further validated by an analysis of the eels' mitochondrial cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (16S rRNA) sequences. Previous studies had reported the occurrence of the tropical eel species *A. marmorata* in Peninsular Malaysia without validating the identification genetically, and after re-examination a number of key morphological characteristics of a preserved sample of *A. marmorata*, the sample from Peninsular Malaysia was identified as *A. bengalensis bengalensis*. This is the first record of the occurrence of *A. bengalensis bengalensis* in Malaysian waters that was confirmed by both morphological and molecular genetic analyses. Although one sample was identified as *A. borneensis* on key morphological characteristics, molecular genetic analyses showed that the sample was actually *A. bicolor bicolor*. These results indicate the difficulty of accurately identifying tropical eels solely on morphological analyses due to the sympatric distribution of a number of closely related eels.

Resumen: Las anguilas anguílidas que se encuentran en Malasia peninsular fueron identificadas, por medio de análisis morfológicos como *Anguilla bengalensis bengalensis* y *A. bicolor bicolor*; dicha identificación fue validada, además, por medio de análisis de secuencias de la subunidad I de la oxidasa del citocromo mitocondrial (COI) y del ARN ribosomal 16S (rARN 16S) de las anguilas. Estudios previos han reportado la presencia de la especie de anguila tropical *A. marmorata* en Malasia peninsular sin validar genéticamente la identificación, y después de reexaminar un número de características morfológicas clave de una muestra preservada de *A. marmorata*, la muestra de Malasia peninsular fue identificada como *A. bengalensis bengalensis*. Éste es el primer registro de la presencia de *A. bengalensis bengalensis* en aguas malayas confirmado tanto por análisis morfológicos como de genética molecular. Aunque una muestra fue identificada como *A. borneensis* a partir de características morfológicas clave, los análisis de genética molecular mostraron que en realidad la muestra correspondía a *A. bicolor bicolor*. Estos resultados muestran la dificultad para identificar con exactitud las anguilas tropicales solamente con base en análisis morfológicos debido a la distribución simpátrica de un número de anguilas relacionadas cercanamente.

Resumo: As enguias encontrados na península da Malásia foram identificadas, por meio de análise morfológica, como sendo a *Anguilla bengalensis bengalensis* e a *A. bicolor bicolor*,

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identificação, que foi ainda validado por uma análise da subunidade da oxidase I (COI) do citocroma mitocondrial das enguias e pelas sequências (16S rRNA) do RNA ribossomal. Estudos anteriores haviam relatado a ocorrência da espécie de enguia tropical *A. marmorata* na península da Malásia sem validar geneticamente a sua identificação, mas após a reexame de uma série de características morfológicas fundamentais de uma amostra preservada de *A. marmorata*, a amostra da península da Malásia foi identificada como sendo a A. bengalensis bengalensis. Este é o primeiro registo da ocorrência de *A. bengalensis bengalensis* em águas da Malásia que foi confirmada na base das características morfológicas e de genética molecular. Apesar de uma amostra ter sido identificada como *A. borneensis*, com base nas características morfológicas, a análise genética molecular revelou que a amostra era efetivamente de A. bicolor bicolor. Estes resultados indicam a dificuldade de identificar com precisão as enguias tropicais com recurso exclusivo à análise morfológica, devido à distribuição simpátrica de um número de enguias estreitamente relacionadas.

Key words: Cytochrome, distribution, Malaysia, molecular, species identification, tropical anguillid eels, 16S rRNA.

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Introduction

The freshwater eels of the genus *Anguilla* Schrank 1798 are widely distributed throughout the world. Nineteen species of *Anguilla* have been reported worldwide, thirteen of which occur in tropical regions (Ege 1939; Watanabe *et al.* 2009). Of the thirteen species found in tropical areas, seven species or subspecies occur in the western Pacific around Indonesia and Malaysia, i.e., *A. celebesensis* Kaup 1856, *A. interioris* Whitely 1938, *A. bengalensis bengalensis* Gray 1831, *A. marmorata* Quoy & Gaimard 1824, *A. borneensis* Popta 1924, *A. bicolor bicolor* McClelland 1844 and *A. bicolor pacifica* (Schmidt, 19280) (Arai *et al.* 1999) (Ege 1939; Castle & Williamson 1974). These eels have a catadromous life history, migrate between inland or coastal growth habitats, and spawn offshore.

Comprehensive studies by Ege (1939) have discussed anguillid species diversity, geographic distribution and abundance in the world and have revealed that the highest diversity of anguillids occurs in central Indonesian waters. However, for Malaysia, there is relatively little information available on various aspects of eel biology including species composition, distribution, life history and migration. Malaysia should not be excluded as a study area, as it is one of the important geographical niches of anguillids; eel biology research in Malaysia could provide details on their species diversity, evolutionary pathway and life history.

According to several past studies, the tropical eel species *Anguilla bicolor bicolor* and *A. marmorata* have been found in Peninsular Malaysia (Ahmad & Lim 2006; Arai *et al.* 2012; Azmir & Samat 2010; Ng & Ng 1989). However, these studies, except for one by Arai *et al.* (2012), did not perform comprehensive identification methods for the anguillid species. The identification of eels at the species level using solely visual observation is known to be difficult because of the similarities and overlapping morphological characteristics in eels, particularly tropical anguillids (Ege 1939; Watanabe *et al.* 2004). To validate the identification of the tropical eel species, it is crucial to utilise both morphological and molecular genetic analyses.

Recently, Arai *et al.* (2012) reported finding the tropical eel species *A. bicolor bicolor* in the western parts of Peninsular Malaysia based on key morphological characteristics. In the present study, we collected anguillid eels from Langkawi Island, Peninsular Malaysia. These eels were subjected to identification using both morphological analyses and mitochondrial cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (16S rRNA) sequence analysis. We are the first to discover *Anguilla bengalensis bengalensis* in Malaysian waters. The present study also highlights the limitation of tropical eel species identification based solely on morphological analyses.

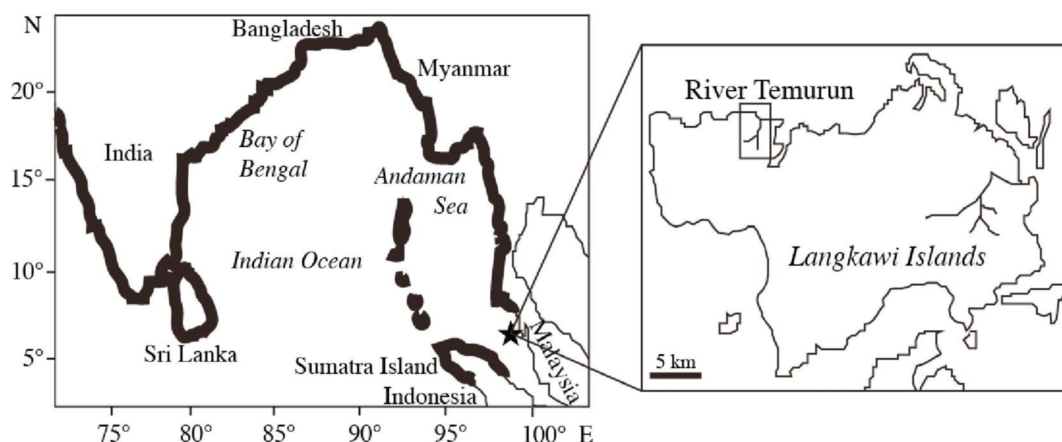

Fig. 1. Sampling site at the Temurun River in Langkawi Islands in the Kedah State of northern Peninsular Malaysia. The distribution of *Anguilla bengalensis bengalensis* (thick lines) determined by Ege (1939) and the finding of *A. bengalensis bengalensis* in Langkawi Island, Peninsular Malaysia in the present study (star).

Material and methods

Fish sample collection

Three anguillid eels were collected using fish traps and angling fishing in the Temurun River of Langkawi Island in the Kedah State of Northern Peninsular Malaysia from May 9 to 13, 2013 (Fig. 1). During the field surveys, we explored other rivers in the island, but anguillids were only found in the Temurun River.

In the present study, a formalined sample collected by Ahmad & Lim (2006) from the Temurun River on April 9th 2003 was subjected to morphological re-examination. The eel was captured in a fish trap set below the Temurun waterfall.

Morphological analysis

The external morphometric characteristics were measured for each sample according to the morphological description of Ege (1939) and Watanabe *et al.* (2004) (Table 1), and, thereafter, the dorsal fins were clipped and preserved in 95 % ethanol for molecular genetic analysis. Given that the total number of vertebrae (TV) is the same in a number of anguillids, the TV may not be a useful tool for the taxonomic identification of anguillids (Ege 1939; Watanabe *et al.* 2004). Thus, vertebrae counting was not conducted in the present study.

Instead, another morphological characteristic was chosen for the identification of eel samples. Based on the anguillid morphological identification keys developed by Ege (1939), the fin difference index (FDI) provides the highest resolution with the least ambiguity when distinguishing eels at the species level. The FDI for the distance between the verticals from the beginning of the dorsal fin (Z) to the anus (ano-dorsal length) relative to the total length (L_T) (Ege 1939), was calculated as follows:

FDI = 100 Z L_T^{-1}.

Anguilla has been clearly divided into four different species groups based on the external morphological characteristics of each species: the first group (four species) has variegated skin with broad maxillary bands of teeth, the second group (four species/subspecies) has variegated skin with narrow maxillary bands of teeth, the third group (six species) has non-variegated skin with a long dorsal fin, and the fourth group (five species/subspecies) has non-variegated skin with a short dorsal fin (Table 2) (Ege 1939; Watanabe *et al.* 2004). Furthermore, geographic distribution has also been used as an important criterion for species identification with the *Anguilla* genus (Ege 1939). Thus, the species of anguillid eels that were collected in this study were first identified using their morphological characteristics and geographic distributions.

DNA extraction

DNA was isolated from dorsal finclips of the three eels using a Gentra Puregene Tissue Kit (QIAGEN, USA), according to the manufacturer's protocol. The quantity and quality of the extracted DNA were estimated using a BioPhotometer Plus spectrophotometer (Eppendorf, Germany) as well as electrophoresis on 1.5 % agarose gel stained with SYBR® Safe (Invitrogen, USA).

Table 1. Biological data of *Anguilla* spp. collected at the Temurun River of Langkawi Islands in Kedah State of northern Peninsular Malaysia. -: Molecular genetic analysis could not be performed because the sample was preserved in formalin.

Sampling date	Body weight (g)	Total length (mm)	Pre-anal length (mm)	Pre-dorsal length (mm)	Fin difference index (%)	Pattern of color marking of skin	Characters of maxillary bands of teeth	Species determined by morphology	Species determined by molecular genetics
9 May 2013	205	491	154	207	10.77	variegated	narrow	*A. bengalensis bengalensis*	*A. bengalensis bengalensis*
11 May 2013	155	462	133	184	11.04	variegated	narrow	*A. bengalensis bengalensis*	*A. bengalensis bengalensis*
13 May 2013	805	732	256	323	9.15	non-variegated	wide	*A. borneensis*	*A. bicolor bicolor*
9 April 2003	1351	831	256	332	9.15	variegated	narrow	*A. bengalensis bengalensis*	-

Table 2. Distinct morphological characteristics classified into four groups by Ege (1939) and Watanabe *et al.* (2004, 2009) with representative geographical distribution range for each anguillid species.

Group	Species	Distinct morphological characters	Geographical distribution range
1	*A. celebesensis*	variegated skin	Jawa, Sumatra Sulawesi island in Indonesia Philippines, New Guinea
	A. interioris	broad maxillary bands of teeth	New Guinea
	A. megastoma	long dorsal fin	Solomon Islands, New Caledonia, Fiji Islands, Cook Islands
	A. luzonensis		Northern Philippines
2	*A. bengalensis bengalensis*	variegated skin	Sri Lanka, Bangladesh, India, Myanmar, Sumatra Island in Indonesia and Andaman Islands
	A. bengalensis labiata	narrow maxillary bands of teeth	Mid-southeastern part of Africa
	A. marmorata	long dorsal fin	Longitudinally from the east coast of Africa to the Marquesas Islands in the southeast Pacific Ocean and as far north as southern Japan
	A. reinhardtii		Easten Australia, Northern New Zealand
3	*A. borneensis*	non-variegated skin	Borneo Island
	A. japonica	long dorsal fin	Japan, China, Korea, Taiwan, Northern Philippines
	A. rostrata		North and South America
	A. anguilla		Europe, North Africa
	A. dieffenbachii		New Zealand
	A. mossambica		Mid-southeastern part of Africa, Madagascar
4	*A. bicolor pacifica*	non-variegated skin	Philippines, Sulawesi Island in Indonesia, New Guinea
	A. bicolor bicolor	short dorsal fin	Africa, India, Sri Lanka, Bangladesh, Mynmar, northwestern Australia, Greater Sunda Islands
	A. obscura		northeastern Australia, New Caledonia, Fiji Islands, Samoa, Tahiti, Cook Islands, Maluku Islands
	A. australis australis		Southeastern Australia, Tasmania
	A. australis schmidtii		New Zealand, New Caledonia, North Nolfork Island

Table 3. Accession numbers in NCBI database for *Anguilla borneensis*, *A. bengalensis bengalensis* and *A. bicolor bicolor*.

Species	Gene	Source of sequences	NCBI Accession Numbers
Anguilla borneensis	16S rRNA	NCBI database	AB188420.1
			AB188418.1
			AB188416.2
			AB188417.1
			AB188414.1
			AB097767.1
			AB097713.1
			AB097711.1
Anguilla bengalensis bengalensis	16S rRNA	Present study	KF182299
			KF182300
	COI		KF182302
			KF182303
Anguilla bicolor bicolor	16S rRNA		KF182301
	COI		KF182304

PCR amplification and sequencing

Two mitochondrial regions were used to confirm the species identity of each sample. A fragment of the 5' end of the cytochrome oxidase subunit I (COI) gene of approximately 701 base pair (bp) in length was amplified using the following universal fish barcoding primers: FishR2-(5′ TCA ACC AAC CAC AAA GAC ATT GGC AC 3′), FishR1- (5′ TAG ACT TCT GGG TGG CCA AAG AAT CA 3′), FishF2-(5′ TCG ACT AAT CAT AAA GAT ATC GGC AC 3′), and FishF1- (5′ ACT TCA GGG TGA CCG AAG AAT CAG AA 3′) (Ward *et al.* 2005). The second amplified region was a 551 bp fragment of the 16S ribosomal RNA (16S rRNA) gene and it was amplified using the following primer pairs: L2510 (5′CGC CTG TTT ATC AAA AAC AT 3′) and H3080 (5′ CCG GTC TGA ACT CAG ATC ACG T 3′) (Palumbi *et al.* 1991). The PCR amplifications were performed in 25 µl reaction volumes containing 1x Invitrogen Platinum Taq Buffer, 1.5 m M $MgCl_2$, 10 pmol of each primer 0.25 mM of each deoxynucleotide triphosphate (dNTPs), 1.5 U of Taq DNA polymerase and 100 ng of genomic DNA. The thermal cycler parameters included an initial denaturation step at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C (for COI) and 55 °C (for 16S rRNA) for 30 s, and elongation at 72 °C for 1 min. The PCR reactions were terminated after the final elongation step at 72 °C for 10 min. To ensure that the reactions yielded amplicons of the appropriate sizes, the amplified fragments were electrophoresed and visualised on 2.0 % agarose gels. Purification of the PCR amplicons was conducted using a QIAquick® PCR Purification Kit (QIAGEN, USA) according to the supplier's protocol. The purified products were labelled using a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems Inc., USA) and sequenced bi-directionally on an ABI PRISM 3730xl Genetic Analyzer following the manufacturer's instructions. Sequence Analysis Software Version 5.2 (Applied Biosystems, Inc.) was used to generate sequence tracefiles and contiguous read lengths.

Sequence analysis

Bi-directional sequences were manually assembled to form contigs using Vector NTI software (Invitrogen Inc., USA). The assembled contigs were end-trimmed using the SeqMan program (DNASTAR Inc., USA) so that only a homologous region remained. The end-trimmed sequences were then compared for percentage similarity with the reference sequences in the NCBI database using BLASTn; then, the end-trimmed sequences were submitted to the NCBI database with the registered accession numbers (Table 3). The nucleotide sequences for *Anguilla bengalensis bengalensis* generated by this study were aligned to the reference sequences with Vector NTI software to produce consensus

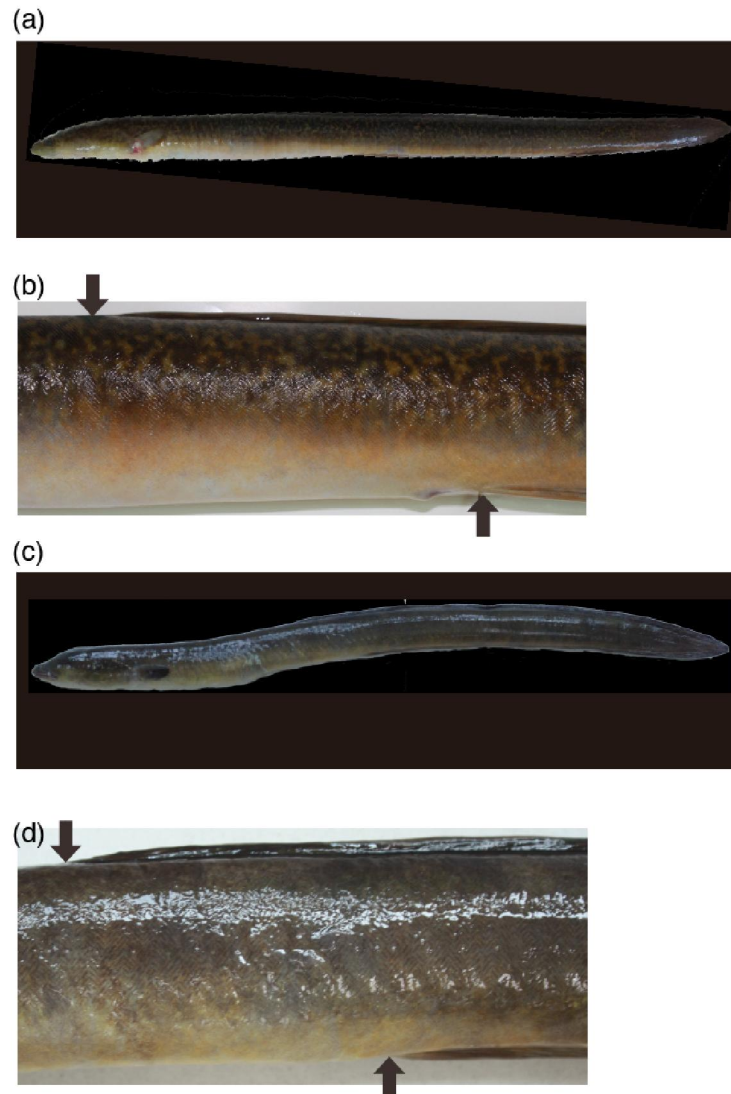

Fig. 2. *Anguilla bengalensis bengalensis* and *A. bicolor bicolor* collected in the Temurun River of the Langkawi Islands in northern Peninsular Malaysia from May 9 to 13, 2013. (a) Total length of *Anguilla bengalensis bengalensis* (492 mm). (b) Length of dorsal and pre-anal fins of *A. bengalensis bengalensis*. (c) Total length of *A. bicolor bicolor* (732 mm). (d) Length of dorsal and pre-anal fins of *A. bicolor bicolor*. Upper arrow indicates origin of dorsal fin and lower arrow indicates origin of pre-anal fin for each species.

sequences (16S rRNA) and consensus barcode sequences (COI) (Wong *et al*. 2011). The edited contigs and consensus sequences were aligned using CLUSTALW. The extent of sequence difference between the species was estimated by averaging the pair-wise genetic distances based on the Kimura-2-Parameter (K2P) distance model (Kimura 1980) using MEGA, version 5.0 (Tamura *et al*. 2011). Sequences of *Anguilla borneensis* (synonym *Anguilla malgumora*) were also retrieved from NCBI databases, and their accession numbers are listed in Table 3. The consensus sequences for *A. borneensis* were subjected to multiple sequence alignment constructions and K2P distance matrix comparisons for the studied species.

Results

Morphological implications

Two of the three samples and a formalined fish had skin with variegated markings (Fig. 2a), narrow maxillary bands of teeth and long dorsal fins (Fig. 2b). The other sample had skin without variegated markings (Fig. 2c) and a long dorsal fin (Fig. 2d).

Table 4. Summary of *Anguilla* spp. identification based on mitochondrial cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (16S rRNA) sequences using BLASTn search from NCBI.

Morphologically analyzed as	NCBI (BLASTn)		
	Species identification	% Max identity	
		COI	16S rRNA
Anguilla bengalensis bengalensis	*Anguilla bengalensis bengalensis*	99	100
Anguilla bengalensis bengalensis	*Anguilla bengalensis bengalensis*	99	99
Anguilla borneensis	*Anguilla bicolor bicolor*	99	100

Two of the three samples and a formalined fish were assigned into the second group of the genus *Anguilla* based on their variegated skin and narrow maxillary bands of teeth (Ege 1939; Watanabe *et al*. 2004). The geographical distribution of anguillids is used in combination with key morphological characteristics to determine the classification of each species into four groups. Within the second group, *A. bengalensis labiata* and *A. reinhardtii* exist in the mid-southeastern region of Africa and eastern Australia and Tasmania, respectively (Ege 1939) (Table 2). Therefore, both of these species were not considered when identifying the samples in the present study. The FDI of the other two species, *A. bengalensis bengalensis* and *A. marmorata* was studied further. According to the key morphological characteristics used for identification (Ege 1939; Watanabe *et al*. 2004), the FDI of *A. marmorata* is in the range of 12 to 20, higher than that of *A. bengalensis bengalensis*, which is in the range of 8 to 14 (Ege 1939; Watanabe *et al*. 2004). Two samples in the present study had FDIs in the range of 9 to 11, which is within the FDI range of *A. bengalensis bengalensis* (Table 1).

One of the samples was assigned into the third group of anguillid eels based on its non-variegated skin, long dorsal fin, and FDI value of 9. Based on the geographical distribution of the anguillid species in the third group, *Anguilla borneensis*, which originated in Borneo Island, was assumed to be the closest species match to the third sample that we found in northern Peninsular Malaysia (Table 2).

Genetic implications

The mitochondrial cytochrome oxidase subunit I (COI) and 16S rRNA region of all samples were successfully amplified using PCR. Table 4 shows the species identity verification of the previously morphologically identified *Anguilla* spp. based on the highest percentage sequence similarities of both genes with their corresponding top matches in the NCBI database. Both mitochondrial regions revealed definitive identity matches in the range of 99 - 100 % for all collected samples with an alignment E-value of 0.0, indicating highly significant similarities. The NCBI reference sequences had high agreement with the samples that were morphologically identified as *Anguilla bengalensis bengalensis*. However, the third sample, which had been morphologically identified as *A. borneensis,* was molecularly identified as *Anguilla bicolor bicolor*. Nevertheless, the identity of the third sample could not be determined conclusively, as precise species identification requires more than a single sample.

Table 5. Pairwise distances between *Anguilla borneensis* (NCBI) and two species of the present study based on 16S rRNA and COI consensus sequences. Genetic distance was denoted by number of base substitutions per site between species (below diagonal) and the standard error estimates are shown above the diagonal. This analysis was conducted using Kimura-2-Parameter Model, which involved complete deletion of all codon positions.

16S rRNA sequences			
Species	1	2	3
1 *Anguilla bengalensis bengalensis*		0.007	0.011
2 *Anguilla bicolor bicolor*	0.024		0.010
3 *Anguilla borneensis*	0.058	0.048	
COI sequences			
Species	1	2	
1 *Anguilla bengalensis bengalensis*		0.01	
2 *Anguilla bicolor bicolor*	0.07		

Consensus sequences and contigs of both mitochondrial genes from *Anguilla bengalensis bengalensis* and *A. bicolor bicolor* were treated as discrete units for estimating the pair-wise level of genetic divergence using the Kimura-2-parameter (K2P) correction model in Table 5 (Nei & Kumar 2000). The K2P distance between these two species

showed relatively low con-specific divergences of 0.07 (COI) and 0.024 (16S rRNA). Accordingly, this low level of genetic divergence was supported by the sister relationship between these two species (Minegishi *et al*. 2005). The genetic divergence between *Anguilla borneensis* and *A. bicolor bicolor* based on the K2P distance matrix generated from 16S rRNA is 0.048.

Discussion

This is the first description of the occurrence and distribution of *Anguilla bengalensis bengalensis* in Peninsular Malaysia as identified by both morphological and genetic analyses. *A. bengalensis bengalensis* is widely distributed in Sri Lanka, Bangladesh, India, Myanmar, Sumatra Island in Indonesia and the Andaman Islands (Ege 1939; Watanabe *et al*. 2004) (Fig. 1). Because there is no aquaculture and no commercial fisheries known to farm eels in Peninsular Malaysia, including the region studied here, Langkawi Island, it seems quite improbable that *A. bengalensis bengalensis* could have been introduced as an exotic species to Peninsular Malaysia. Moreover, there have been no reports of the release of eels into natural waters in Malaysia. All these results suggest that *A. bengalensis bengalensis* is native to this part of Malaysia.

In previous studies, *Anguilla marmorata* was reported to exist in Langkawi Island, Peninsular Malaysia (Ahmad & Lim 2006; Azmir & Samat 2010). Recently, we discovered *A. bicolor bicolor* in the western and northern parts of the Peninsular Malaysia (Arai *et al*. 2012), but *A. marmorata* and *A. bengalensis bengalensis* have only been found in Langkawi Island. After a thorough morphological re-examination by Ahmad & Lim (2006) of one formalined sample of *A. marmorata*, we discovered that the true identify of that particular sample is actually *A. bengalensis bengalensis*. The species misidentification in the previous study may have been due to an insufficient morphological characteristic analysis. In fact, the difficulty in distinguishing both *A. marmorata* and *A. bengalensis bengalensis* is augmented by their overlapping morphological characteristics, which cause further identification ambiguities. Therefore, previous reported *A. marmorata* samples may have actually been *A. bengalensis bengalensis* samples. Thus, comprehensive morphological identification may be strengthened by the integration of molecular marker analyses to further validate the true identity of a species.

Since the genetic divergence between *Anguilla borneensis* and *A. bicolor bicolor* is relatively high, the third sample could be conclusively identified as *A. bicolor bicolor*, not *A. malgumora*. Nevertheless, *A. borneensis* and *A. bengalensis bengalensis* showed a relatively higher level of genetic divergence (0.058) compared to that of *A. borneensis* and *A. bicolor bicolor* (0.048). The inconclusive morphological identification of the third sample is not merely a technical error, but, rather, is due to the inadequacy of the description of the key morphological characteristics of *A. borneensis*. Additionally, misidentification using key morphological characteristics has been reported for *A. celebesensis* and *A. interioris* (Aoyama *et al*. 2000). Thus, an extensive morphological study might be required for *Anguilla borneenis* using more samples from different sampling locations.

This study highlights the first record of the occurrence of *Anguilla bengalensis bengalensis* in Malaysian waters. Although this species is widely distributed throughout the Indo-Pacific region, little or almost no information is available on the life history and migration pattern of this species. Species diversity dynamics are a result of historical, ecological and evolutionary factor and these vary spatially and temporally (Nyabi *et al*. 2014). There is also a lack of this information for other tropical anguillid eel species. Therefore, further studies regarding the distribution, biology and ecology of *Anguilla* using valid species identifications methods are crucial for gaining understanding of the species composition and the diversity of tropical anguillid eels.

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