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Occurrence of the tropical eels *Anguilla bengalensis bengalensis* and *Anguilla bicolor bicolor* in Peninsular Malaysia and implications for eel taxonomy

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The anguillid eels found in Peninsular Malaysia were identified using a morphological analysis and that identification was further validated as Anguilla bengalensis bengalensis *and* Anguilla bicolor bicolor *by an analysis of the eels' mitochondrial cytochrome oxidase subunit I (COI) sequences. Because of the difficulty of accurately identifying tropical eels solely on morphological analyses, previous studies had reported the occurrence of the tropical eel species* Anguilla marmorata *in Peninsular Malaysia. This study suggests the occurrence of* Anguilla bengalensis bengalensis *in Malaysian waters, confirmed by both morphological and molecular genetic analyses.* Anguilla bicolor *is further confirmed as the subspecies of* Anguilla bicolor bicolor *by molecular genetic analyses. The present study also suggests that accurate tropical eel species identification requires validation by molecular genetic analysis after a morphological observation.*

INTRODUCTION

Freshwater eels of the genus *Anguilla* have a catadromous life history and are widely distributed throughout the world. Sixteen species, of which three have been divided into subspecies 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. *Anguilla celebesensis* Kaup 1856, *Anguilla interioris* Whitely 1938, *Anguilla bengalensis bengalensis* Gray 1831, *Anguilla marmorata* Quoy & Gaimard 1824, *Anguilla borneensis* Popta, 1924, *Anguilla bicolor bicolor* McClelland 1844 and *Anguilla bicolor pacifica* (Schmidt, 1928) (Ege, 1939; Castle & Williamson, 1974; Arai *et al.*, 1999). Molecular phylogenetic research on freshwater eels has revealed that tropical eels are the most basal species originating in the Indonesia and Malaysia regions and that freshwater eels radiated out from the tropics to colonize the temperate regions (Minegishi *et al.*, 2005). Recently, various aspects of the biology of tropical eel species in Indonesian waters, including species composition, distribution, life history and migration, have been gradually accumulated (e.g. Arai *et al.*, 1999, 2001, 2002, 2003; Sugeha, *et al.*, 2001; Chino & Arai, 2010a, b; Arai & Chino, 2012, 2013; Arai,

2014a, b). However, for Malaysia, there is relatively little information available on such aspects of eel biology. Malaysia should not be excluded as a study area, as it is one of the important geographical niches for 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 *Anguilla marmorata* have been found in Peninsular Malaysia (Ng & Ng, 1989; Ahmad & Lim, 2006; Azmir & Samat, 2010; Arai *et al.*, 2012). However, these studies did not perform the comprehensive identification methods required for identifying 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 in tropical anguillids (Ege, 1939; Watanabe *et al.*, 2004). To validate the identification of the tropical eel species, it is crucial to utilize both morphological and molecular genetic analyses.

Recently, Arai *et al.* (2012) reported the tropical eel species *Anguilla bicolor bicolor* in the western parts of Peninsular Malaysia based on key morphological characteristics. In the present study, we collected anguillid eels from Penang Island, Peninsular Malaysia. These eels were subjected to identification using both morphological analyses and mitochondrial cytochrome oxidase subunit I (COI) sequence analysis. The present study suggests the limitations of tropical eel species identification based solely on morphological analyses and the need for further molecular genetic analysis for the accurate validation of a species.

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MATERIALS AND METHODS

Fish and morphological observation

Eight anguillid eels were collected using fish traps and angling fishing in the Kongsi River in Penang Island, Peninsular Malaysia, from 11 to 14 May 2014 (Fig. 1). 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 96% ethanol for molecular genetic analysis.

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:

$$FDI = 100 \; ZL_T^{-1}. \qquad (1)$$

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 (Ege, 1939; Watanabe *et al.*, 2004).

Molecular genetic analysis

The mitochondrial gene, cytochrome oxidase *c* subunit 1 (COI), was studied for the identification of the samples.

DNAs were extracted from the dorsal fin, following the protocol of INTRON G-Spin^{TM} Total DNA Extraction Kit (Cat. No.: 17045). The DNAs were amplified using polymerase chain reaction (PCR) according to the thermal cycling conditions modified from Ward *et al.* (2005). An approximate of 700 base pairs of the COI gene were amplified using reagents from INTRON i-Taq^{TM} Plus (Cat. No.: 25152). The fish primers F1 (5′-TCA ACC AAC CAC AAA GAC ATT GGC AC-3′) and R2 (5′-ACT TCA GGG TGA CCG AAG AAT CAG AA 3′) were employed in this process

(Ward *et al.*, 2005). The PCR was performed in a volume of 25 μl for each sample, with the reagent proportions shown in Table 2. The PCR was initiated at 94°C for 2 min, followed by the denaturation at 94°C for 30 s, the annealing at 53–54°C for 30–40 s and an extension at 72°C for one minute. These steps were repeated for 29–34 cycles, followed by a final extension at 72°C for 10 min. The PCR products were eventually kept cool at 4°C.

All PCR products were visualized with 2% agarose gel electrophoresis stained with ethidium bromide. Successful PCR products were sent to First BASE Laboratories Sdn. Bhd. for purification and sequencing.

DNA sequences obtained were aligned in MEGA 6, followed by BLAST for identification on a GenBank sequences database produced by the National Centre of Biotechnology Information (NCBI).

RESULTS

Morphological implications

Five of the eight samples had skin with variegated markings, narrow maxillary bands of teeth and long dorsal fins. The other samples had skin without variegated markings and short dorsal fins (Table 1).

Five of the eight samples 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). Within the second group, *Anguilla bengalensis labiata* and *Anguilla reinhardtii* exist in the mid-south-eastern 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, *Anguilla bengalensis bengalensis* and *Anguilla marmorata*, was studied further. According to the key morphological characteristics used for identification (Ege, 1939; Watanabe *et al.*, 2004), the FDI of *Anguilla marmorata* is in the range of 12–20, higher than that of *Anguilla bengalensis bengalensis*, which is in the range of 8–14 (Ege, 1939; Watanabe *et al.*, 2004). Five samples in the present study had FDIs in the range of 11–12 (Table 1) which overlapped within the FDI range of *Anguilla bengalensis bengalensis* and *Anguilla marmorata*.

Three of the samples were assigned into the fourth group of anguillid eels based on their non-variegated skins and short
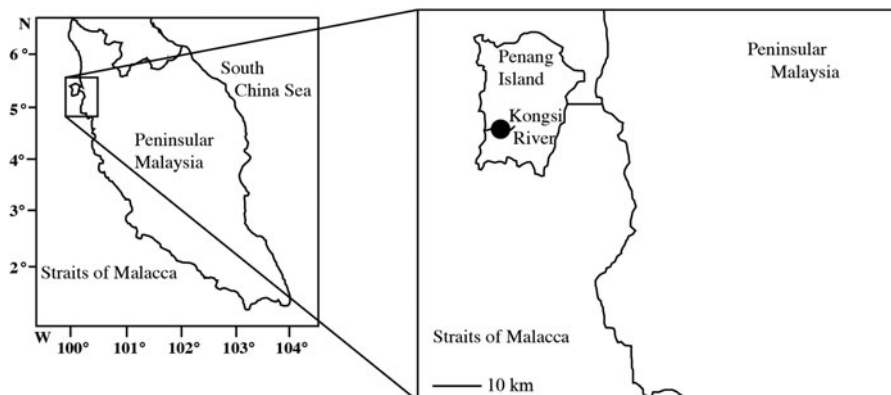

Fig. 1. Sampling site at Kongsi River, Penang Island, Peninsular Malaysia.

Table 1. Biological data of *Anguilla* spp. collected in the Kongsi River, Penang Island, Peninsular Malaysia.

Sampling date	Body weight (g)	Total length (mm)	Preanal length (mm)	Predorsal length (mm)	Fin difference index (%)	Patten of colour marking of skin	Characters of ano-dorsal length	Species determined by morphology	Species determined by molecular genetics
11 May 2014	324	543	216	156	11.0	Variegated	Long finned	*Anguilla bengalensis bengalensis* or *Anguilla marmorata*	*Anguilla bengalensis bengalensis*
11 May 2014	437	618	259	191	11.0	Variegated	Long finned	*Anguilla bengalensis bengalensis* or *Anguilla marmorata*	*Anguilla bengalensis bengalensis*
11 May 2014	363	594	257	259	12.3	Variegated	Long finned	*Anguilla bengalensis bengalensis* or *Anguilla marmorata*	*Anguilla bengalensis bengalensis*
13 May 2014	1070	832	361	257	11.1	Variegated	Long finned	*Anguilla bengalensis bengalensis* or *Anguilla marmorata*	*Anguilla bengalensis bengalensis*
13 May 2014	763	768	337	361	11.8	Variegated	Long finned	*Anguilla bengalensis bengalensis* or *Anguilla marmorata*	*Anguilla bengalensis bengalensis*
11 May 2014	324	560	236	228	1.4	Non-variegated	Short finned	*Anguilla bicolor bicolor* or *Anguilla bicolor pacifica*	*Anguilla bicolor bicolor*
11 May 2014	388	604	254	234	3.3	Non-variegated	Short finned	*Anguilla bicolor bicolor* or *Anguilla bicolor pacifica*	*Anguilla bicolor bicolor*
11 May 2014	69	353	149	148	0.3	Non-variegated	Short finned	*Anguilla bicolor bicolor* or *Anguilla bicolor pacifica*	*Anguilla bicolor bicolor*

Table 2. Volume (µl) and concentration of reagents used for PCR.

Reagents	Volume (µl)
Buffer (10× Mg^{2+} free)	2.5
MgCl$_2$ (25 mM)	2
dNTP (2.5 mM)	1 – 2
F1 (10 µm)	0.5 – 1.0
R2 (10 µm)	0.5 – 1.0
Taq DNA polymerase (5 U µl^{-1})	0.25 – 0.50
DNA	2
ddH$_2$O	14 – 16.25

dorsal fins. Based on the geographical distribution of the anguillid species in the fourth group, those samples could be confirmed as either *Anguilla bicolor bicolor* or *Anguilla bicolor pacifica*. These two subspecies, however, are almost identical in their morphological characteristics (Ege, 1939; Watanabe *et al.*, 2004) and only the genetic characteristics enable them to be divided into different subspecies.

Genetic implications

The mitochondrial cytochrome oxidase *c* subunit I (COI) of all samples was successfully amplified using PCR. The mitochondrial region revealed definitive identity matches in the range of 96–100% for all collected samples, indicating highly significant similarities. The NCBI reference sequences had a high agreement with the samples that were identified as *Anguilla bengalensis bengalensis* for five variegated long-finned eels and *Anguilla bicolor bicolor* for three short-finned eels.

The DNA sequences of five haplotypes of *Anguilla bengalensis bengalensis* were deposited in the GenBank sequences database with the accession numbers KM875498 to KM875502, whilst the DNA sequences of three haplotypes of *Anguilla bicolor bicolor* were deposited in the same database with the accession numbers KM875503 to KM875505.

DISCUSSION

This is the concrete description of the occurrence and distribution of *Anguilla bengalensis bengalensis* and *Anguilla bicolor bicolor* in Peninsular Malaysia as identified by both morphological and molecular genetic analyses.

Anguilla 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). In previous studies, *Anguilla marmorata* was reported to exist in Peninsular Malaysia (Ahmad & Lim, 2006; Azmir & Samat, 2010). After a thorough morphological re-examination by Ahmad & Lim (2006) of one formalined sample of *Anguilla marmorata*, the true identify of that particular sample was actually *Anguilla bengalensis bengalensis* based on the value of 9 in the FDI (Arai, 2014b). The species misidentification in the previous study may have been due to an insufficient morphological characteristic analysis. In fact, the difficulty in distinguishing both *Anguilla marmorata* and *Anguilla bengalensis bengalensis* is augmented by their overlapping morphological characteristics, which cause further identification ambiguities. The FDI values in the long-finned eels were 11–12 and could not be used to identify samples morphologically in the present study due to

the overlapping geographical distribution of several eel species and proximity in the tropical region. Thus, comprehensive morphological and molecular genetic identifications are needed for the tropical eel species of tropical regions to further validate the true identity of a species.

Anguilla bicolor has the second widest geographic distribution of any species of the genus *Anguilla,* except for *Anguilla marmorata. Anguilla bicolor* is distributed from the eastern coast of Africa, through the Indonesian Seas to New Guinea, adjacent to the Pacific Ocean (Ege, 1939). Ege (1939) divided *Anguilla bicolor* into the two subspecies *Anguilla bicolor bicolor* and *Anguilla bicolor pacifica,* primarily because of the slight difference in their means and ranges of total number of vertebrae. The distribution range of *Anguilla bicolor bicolor* includes the coast of Africa, India, Sri Lanka, Bangladesh, Myanmar, north-western Australia and the Greater Sunda Islands, and the distribution range of *Anguilla bicolor pacifica* includes the coast of China, Vietnam, the Philippines, Borneo Island, Sulawesi Island and New Guinea Island. Recently, Arai *et al.* (2012) found a new distribution range of *Anguilla bicolor bicolor* in Peninsular Malaysia using morphological characteristics, and the present study also supports the occurrence of *Anguilla bicolor bicolor* in Peninsular Malaysia by means of molecular genetic signatures. Malaysia, however, locates the boundary or overlapping parts of the ranges of the two subspecies. Thus, both morphological and molecular genetic identifications are needed for accurate species identification.

This study highlights the occurrence of *Anguilla bengalensis bengalensis* and *Anguilla bicolor bicolor* in Malaysian waters. Although those species are widely distributed throughout the Indo-Pacific region, little or almost no information is available on the life history and migration pattern of this species. Therefore, further studies regarding the distribution, biology and ecology of tropical anguillid eels using valid species identifications methods are needed to understand their mysterious life history.

ACKNOWLEDGEMENTS

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