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Using DNA barcodes based on mitochondrial *COI* and *16S rRNA* genes to identify *Anguilla* eels in Thua Thien Hue province, Vietnam

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ABSTRACT. In Vietnam, *Anguilla* eels have been documented before, but their species composition and biological characteristics are still relatively unknown. Research on the molecular phylogeny of *Anguilla* has been limited, and identification of the species of eels was usually based on morphological characteristics. This is the first time both cytochrome c oxydase subunit I (COI) and 16S ribosomal RNA (rRNA) genes were used to identify *Anguilla* eels in water bodies, where the eels can migrate downstream and upstream during different stages of the life cycle, in Thua Thien Hue province, Vietnam. Based on DNA sequence analysis of six specimens, two *Anguilla species* (including subspecies) were confirmed as *A. marmorata* and *A. bicolor pacifica*, which had not been identified based on morphological characteristics in previous analyses.

Phylogenetic analysis showed that the *Anguilla* genus in Thua Thien Hue province belongs to the Indo-Pacific lineage and helped build phylogenetic trees generated from *mt*DNA data from GenBank for these eels.

Key words: *Anguilla*; DNA barcode; *CO*I; 16*S* rRNA; Thua Thien Hue province; Vietnam

INTRODUCTION

Anguilla has a catadromous life-history strategy (Arai, 2016), migrating between freshwater growth habitats and offshore spawning areas (Arai and Chino, 2018) with distances from several hundred to thousands of kilometers (Arai, 2014). Among the 16 species and 3 sub-species of *Anguilla* genus that have been identified worldwide (Ege, 1939; Watanabe, 2003), five species, *A. nebulosa* (McClelland, 1844), *A. japonica* (Temminck & Schlegel, 1984), *A. marmorata* (Quoy & Gaimard, 1824), *A. celebensis* (Kaup, 1856), and *A. bicolor pacifica* (Schmidt, 1928; Phung, 2001) have been identified in Vietnam. Central Vietnam has three species: *A. marmorata*, *A. bicolor pacifica*, and *A. japonica* (Nguyen et al., 2018). In the water bodies in Thua Thien Hue province, two species have been identified, *A. marmorata* and *A. bicolor* (Phu and Dang, 2008; Phu and Ha, 2008; Hoang and Duc, 2012) based on morphological characteristics alone. Eel species identification is usually based on morphological characters of *Anguilla*. However, the identification of eel species using morphology is complicated because of the similarity and overlap of morphological features, especially for *Anguilla* spp. in the tropics (Arai, 2016). Therefore, the application molecular markers in determining species composition of eels in such regions is warranted.

Recently, the use of DNA barcodes to identify species has been researched by scientists around the world and is proving invaluable for understanding species boundaries, community ecology, functional trait evolution, trophic interaction, and biodiversity conservation (Kress et al., 2015). Compared to morphological and chemical indicators, DNA identification is more accurate and does not depend on any subjective factors. For animals, scientists often recommend using markers located on the mitochondrial genome, such as the *cyt b* gene, the *CO*I gene, and the *D-loop* region (Hebert et al., 2003; Hebert and Barrett, 2005). In the present study, we collected *Anguilla* eels from the waters of Thua Thien Hue province, Vietnam. This is the first-time molecular markers have been used to determine the species composition of *Anguilla* eels in Thua Thien Hue province, Vietnam. This study was designed to confirm the presence of previously recognized eel species in Thua Thien Hue province, Vietnam and examine the limitations of identifying tropical eels based only on morphological analysis.

MATERIAL AND METHODS

Animal preparation and sampling

Six natural specimens of *A. marmorata* in yellow stage (non-migratory) and sliver stage (migratory) were collected from five localities, from September 2018 to March 2019

(Figure 1 and Table 1). Tissue from the adductor muscle was dissected from fresh specimens, preserved in 95% ethanol, and frozen at -80°C until DNA extraction.

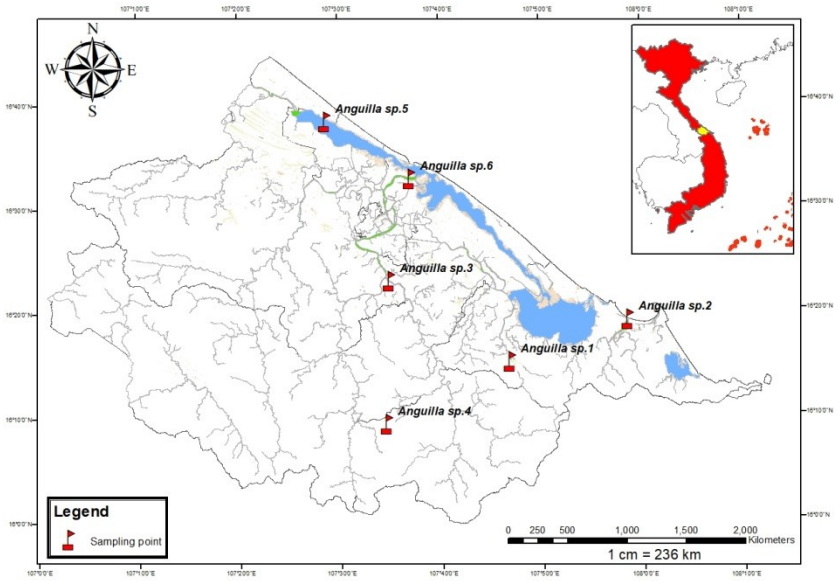

Figure 1. Location of sampling points

Table 1. Information for *Anguilla* spp. collected in Thua Thien Hue, Vietnam.

Sample	Location of sampling site	Date of sample collection	Total length (mm)	Body weight (g)	GenBank accession numbers	
					COI	*16S rRNA*
Angilla sp.1	Truoi dam	15/01/2019 Non-migratory	255.0	34.0	MN067935	MN633320
Angilla sp.2	Bu Lu river	15/10/2018 Non-migratory	362.0	89.0	MN067961	MN633346
Angilla sp.3	Thao Long dam	19/12/2018 Migratory	1080.0	3200.0	MN067930	MN633315
Angilla sp.4	Nam Dong	08/02/2019 Non-migratory	405.0	133.1	MN067945	MN633330
Angilla sp.5	Phong Dien	27/09/2018 Migratory	540.0	388.2	MN067958	MN633343
Angilla sp.6	Thao Long dam	22/11/2018 Migratory	613.0	491.0	-	-

Morphological analysis

The external morphological characteristics were measured for each specimen according to the morphological description by Ege (1939) and Watanabe et al. (2004). The morphological characteristics used included TL (total length); PA (preanal length); HL (head length); PD (the predorsal length); TR (length of the trunk, calculated as the PA minus HL); AD (the distance between the verticals through the anus and origin of the dorsal fin); PDH (PD minus HL) (Watanabe et al., 2004). Six of the 14 taxonomic characters used by Ege (1939) were analyzed in this study, including color marking, dentition, the

proportions of preanal length to total length (PA/TL), preanal length without head to total length (TR/TL), the distance between verticals through anus and origin of the dorsal fin to total length (AD/TL), predorsal length without head length to total length (PDH/TL), and head length to total length (HL/TL) (Ege, 1939; Watanabe et al., 2004).

DNA extraction and sequence analysis

Total genomic DNA was extracted by the method descriped by Kumar et al. (2007) with a modification. The PCRs were carried out using two primer pairs: A.marFw-1-5'GCACTAAGCTTCTAATCCG3' and A.marRv-1-5' GATGATTATTGTGGCAGAAG3' (Huyen and Linh, 2020) for amplification of the *CO*I gene; L2510 (5′CGC CTG TTT ATC AAA AAC AT3′) and H3080 (5′CCG GTC TGA ACT CAG ATC ACG T3′) (Palumbi et al., 1991) for amplification of 16*S rRNA* gene. The amplification reactions were performed in a total volume of 35 µL. Reaction components: 1 µL DNA, 1 µL F-primer (10 mM), 1 µL R-primer (10 mM), 7 µL PCR buffer (10X), 0.5 µL dNTP (10 mM), 0.2 µL *Taq*-polymerase (5 UI/ µL), 1 µL F-primer (10 mM), 1 µL R-primer (10 mM) and sterile distilled water addition to attain volume 35 µL. PCR amplification was performed according to the thermal cycle: 5 °C / 5 minutes; followed by 30 cycles: 95°C / 45 seconds, 51°C (for *CO*I) and 55°C (for *16S rRNA*) / 30 seconds, and 72°C / 1 minute; The last extension was 72^{o}C / 7 minutes on the MJ-MiniTM Persanol Thermal Cycler (Bio-Rad machine. The PCR products were purified by the Isolate II PCR kit (Bioline). These PCR products were then sequenced directly by the dideoxy terminator method on ABI PRISM® 3100 Avant Genetic Analyzer (Applied Biosystems) at Maccrogen company, Korea.

The nucleotide sequences were manually edited and assembled using the ClustalW program (Larkin at el., 2007) on the BioEdit 7.0.5 software (Hall, 1999). The end trimmed sequences were then compared for percentage similarity with the reference sequences in the NCBI database using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST); then, they were submitted to the NCBI database with the registered accession numbers (Table 2). The extent of sequence difference between the species was estimated by averaging the pairwise genetic distances based on the Kimura2-Parameter (K2P) distance model (Kimura, 1980) using MEGA X software (Kumar et al., 2018). Phylogenetic trees showing genetic relationships were constructed using MEGA X software, based on the Neighbor Joining method (Felsenstein, 1985), with a bootstrap value of 1000 replicates.

RESULTS

Morphological implications

Five of the six samples collected had skin with variegated markings, narrow maxillary bands of teeth, and long dorsal fins. The other sample had non-variegated skin markings, wide maxillary bands of teeth, and a long dorsal fin (Table 2). They were classified as group 2 and group 4 of the four of Anguilla eels described by Ege (1939) and Watanabe et al. (2004), respectively. Based on the datasheets of PA/TL (%), HL/TL (%), TR/TL (%), AD/TL (%), PDH/TL (%) for Anguilla ssp. *in Thua Thien Hue province, Vietnam (Table 2) and the* descriptions by Ege (1939) and Watanabe et al. (2004), five samples (Anguilla sp.1 to Anguilla sp.5) belonged to A. marmorata and Anguilla sp.6 belonged to A. bicolor (Table 2).

Table 2. Morphological characteristics of Anguilla spp. collected in Thua Thien Hue, Vietnam.

Sample	*Anguilla* sp.1	*Anguilla* sp.2	*Anguilla* sp.3	*Anguilla* sp.4	*Anguilla* sp.5	*Anguilla* sp.6
W (g)	34.0	89.0	3,200.0	133.1	388.2	491.0
TL (mm)	255.0	362.0	1,080.0	405.0	540.0	613.0
HL (mm)	33.0	49.0	160.0	55.0	75.0	80.0
PD (cm)	6.5	8.4	31.0	11.0	15.0	27.0
PA (mm)	97.0	152.0	500.0	190.0	260.0	270.0
AD (mm)	42.0	59.0	190.0	80.0	110.0	0.0
TR (mm)	64.0	103.0	340.0	135.0	155.0	190.0
PDH (mm)	32.0	35.0	150.0	55.0	75.0	190.0
PA/TL(%)	39	42	46	47	48	44
HL/TL(%)	12.9	13.5	14.8	13.6	13.9	13.0
TR/TL(%)	25.1	28.5	31.5	33.3	28.7	30.1
AD/TL(%)	16.5	16.3	17.6	19.7	20.4	0.0
PDH/TL (%)	12.5	9.7	13.8	13.6	13.9	30.9
Color marking	Variegated	Variegated	Variegated	Variegated	Variegated	Non-variegated
Dentition	Narrow	Narrow	Narrow	Narrow	Narrow	Wide
Species determined by morphology	*A. marmorata*	*A. marmorata*	*A. marmorata*	*A. marmorata*	*A. marmorata*	*A. bicolor*
Species determined by molecular genetics	*A. marmorata*	*A. marmorata*	*A. marmorata*	*A. marmorata*	*A. marmorata*	*A. bicolor pacifica*

W = weight; TL = total length; HL = head length; PD = predorsal length; PA = preanal length; TR = trunk length; AD = distance between the verticals through the anus and origin of the dorsal fin; PDH = (PD minus HL).

Genetic implications

In this study, the gene segments of Cytochrome c oxydase subunit *I (*CO*I) and the* 16S rRNA *region of all samples were successfully amplified. Table 3 showed the summary of identification for* Anguilla *spp. in Thua Thien Hue* province using BLASTn search from NCBI based on the highest percentage of matches of the two gene segments (COI and 16S rRNA) in the NCBI database. Both mitochondrial regions revealed definitive identity matches in the range of 99.53 – 99.88 % 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 A. marmorata. However, Anguilla sp.6 was molecularly identified as A. biocolor pacifica, which had been morphologically identified as A. bicolor (Table 3). This result has confirmed that the sub-species of A. bicolor found in Thua Thien Hue, Vietnam is A. bicolor pacifica.

Table 3. The results of *Anguilla* ssp. identification by *CO*I and *16S rRNA* sequences using BLASTn search from NCBI.

Morphological analysis	**Species identification**	***COI***		***16S rRNA***	
		GenBank	**% Max identity**	**GenBank**	**% Max identity**
A. marmorata	*A. marmorata*	AP007242.1 HQ141374.1	99.88 99.64	AB278871.1	99.84
A. bicolor	*A. bicolor pacifica*	AP007237.3	99.53	AP007237.3 AB278743.1	99.84 99.69

Phylogenetics

Consensus sequences and contigs of both mitochondrial genes from A. marmorata and A. bicolor pacifica were treated as discrete units for estimating the pairwise level of

genetic divergence using the Kimura – 2 – parameter (K2K) correction model (Nei and Kumar, 2000; Arai and Wong, 2016) (Table 3). The K2K distances between A. marmorata individuals showed a low relative con-specific divergence of 0.0172 (COI) and 0.0088 (16S rRNA), respectively. These values for A. bicolor pacifica were 0.000 for both the COI gene and 16S rRNA gene. Besides, the genetic differences between the two populations A. marmorata and A. bicolor pacifica in Thua Thien Hue base on K2P distance matrix created from COI and 16S rRNA were 0.0640 and 0.0145, respectively (Table 4). Accordingly, the low level of genetic differences has shown a very close relationship of these individuals between themselves and populations (Minegishi et al., 2005; Arai and Wong, 2016).

Table 4. The genetic distance between two species of *Anguilla* ssp. in Thua Thien Hue province, Vietnam based on the K2P distance matrix of *COI* and *16S rRNA* genes.

Species	*COI*		*16S rRNA*	
	A. marmorata	*A. bicolor pacifica*	*A. marmorata*	*A. bicolor pacifica*
A. marmorata	0.0172	0.0172	0.0088	0.0145
A. bicolor pacifica	0.0640	0.000	0.0145	0.000

Based on the sequences of COI and 16S rRNA gene segments of Anguilla ssp. individuals in Thua Thien Hue and the reference sequences taken from GenBank (Table 2), phylogenetic trees were built using Mega X software (Figure 2 and Figure 3). Accordingly, the phylogenetic tree of Anguilla genus distributed in Thua Thien Hue was divided into two branches. The first branch included individuals of the A. marmorata population; they were closely related and had very low genetic distance (high degree of similarity) with two control individuals from GenBank: AP007242.1 and HQ141374.1 (COI) in Figure 2 and AP2878871.1 (16S rRNA) in Figure 3, with bootstrap support rate up to 100%. The other branch included A. bicolor pacifica and AP007237.1 for COI (Figure. 2), and AB278743.1 and AP007237.3 for 16S rRNA (Figure. 3) with a minimal genetic distance (high level of similarity).

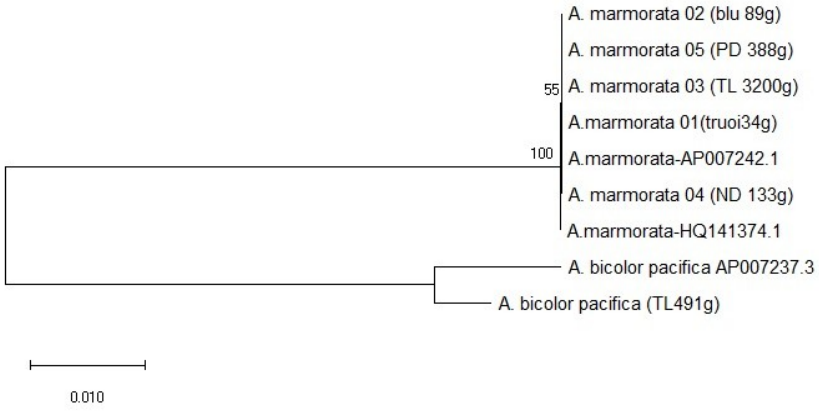

Figure 2. Phylogenetic trees of *Anguilla* ssp. in Thua Thien Hue, Vietnam base on the *COI* segment sequence.

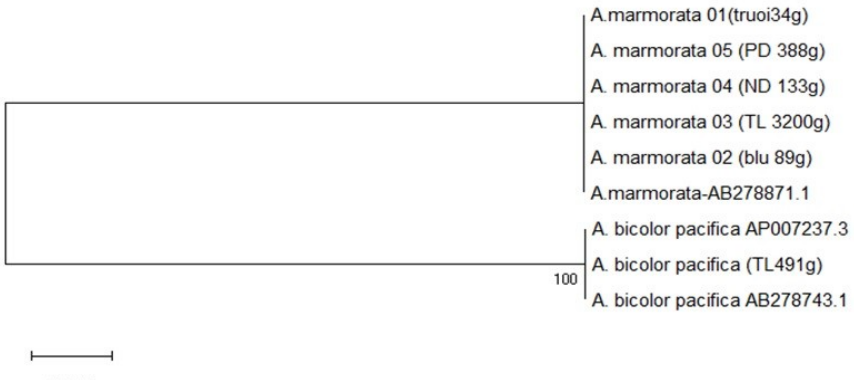

Figure 3. Phylogenetic trees of *Anguilla* ssp. in Thua Thien Hue, Vietnam base on the sequence of *16S rRNA* segment.

DISCUSSION

The identification of *Anguilla* species by morphological indicators are usually the fastest means (Arai, 2016). However, the similarities and overlapping morphological characteristics in eels, particularly tropical *Anguilla* (Watanabe, 2003; Arai, 2016) along with that, the change in their morphology through the stages of development (Watanabe et al., 2003; 2004) made identification of eels at the species level using solely visual observation difficult (Watanabe et al., 2004; 2005; Arai, 2016). In previous studies, the *Anguilla* eel was identified in Thua Thien Hue, Vietnam (Huyen and Phu, 2014). *Anguilla marmorata* is considered to be a widely distributed species, with high economic value (Huyen and Linh, 2019). The species composition of *A. bicolor* has been determined to the species level, but its subspecies have not been determined by morphological analysis (Phu and Dang, 2008; Phu and Ha, 2008; Hoang and Duc, 2012). Therefore, techniques based on DNA analysis were highly effective methods of classifying, identifying, and studying the genetic diversity of eels of *Anguilla* genus (Watanabe et al., 2005; Arai et al., 2020). In this study, this is the first description of two species of *Anguilla* eel, *A. marmorata* and *A. bicolor pacifica* in Thua Thien Hue province, Vietnam, by both morphological and molecular markers. It corresponds to the research result of Nguyen et al. (2018) on the composition of *Anguilla* sp. in central Vietnam by *CO*I gene segment with three species identified as *A. maromorata*, *A. bicolor pacifica,* and *A. japanica*.

Recently, scientists have classified eels based on sequencing of *CO*I and *16S rRNA* genes, which have become effective markers for identifying and studying the relationships among species in the region. Arai et al., (2015; 2016) analyzed *CO*I and *16S rRNA* sequences to identify two species of eel of the *Anguilla* genus in Malaysia. The results identified two species *A. marmorata* and *A. bengalensis bengalensis*, in stark contrast to the identifications based on morphological analysis of *A. celebsensis* and *A. marmorata* (Arai et al., 2015). Based on morphological and molecular markers (*CO*I and *16S rRNA*), Arai et al., (2016), for the first time, *A. bengalensis bengalensis* was one of two species identified in Malaysian coastal (*A. bengalensis bengalensis* and *A. bicolor bicolor*) (Arai and Wong, 2016). In 2017, scientists used morphological analysis and further analysis of *16S rRNA*

sequences to confirm that *A. marmorata* is found in Malaysia (Kadir et al., 2017) and Sabah, Borneo Island (Wong et al., 2017). Hewavitharane et al. (2017) also identified two species, of *marmorata* and *A. megastoma*, and *A. obscura* in the western South Pacific; and three species of *Anguilla* eel in Aceh Waters, Indonesia: *A. marmorata*, *A. bicolor bicolor*, and *A. bengalensis bengalensis* (Muchlisin et al., 2017) using DNA barcode technology based on the *CO*I gene segment.

In 2020, Arai et al. (2020) collected *Anguilla* eels in Southeast Asian waters, i.e. Malaysia, Thailand and Vietnam to identify using both morphological analyses and mitochondrial cytochrome oxidase subunit I (COI) sequence analysis. The molecular phylogenetic tree and the haplotype network of *A. marmorata* in Malaysia, Thailand and Vietnam suggested that the eel might be transported from the Western North Pacific spawning area and propose possible dispersion and migration of *A. marmorata* into Southeast Asian waters. In Thua Thien Hue province, DNA barcode techniques used to analyze molecular characteristics and build phylogenetic based on the *CO*I segment showed that separation of the *A. marmorata* population is guided by the migration process and specific ecological parameters (Huyen and Linh, 2020) and their genetic evolution occurs in the direction of random population expansion (Linh and Huyen, 2020).

CONCLUSIONS

This study was made using molecular markers to determine the composition of species and genetic diversity of Anguilla eels in Thua Thien Hue province, Vietnam, including A. marmorata and A. bicolor pacifica and assessed the relationship between the population of eels in Southeast Asian and other populations in the world. In the future, the DNA barcode and eDNA techniques study should be used more for studying Anguilla eels to better define and understand natural populations that may be in danger of decline.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animals and samples collected conformed to international, national, and regional and Institutional guidelines for animal care and rules in Vietnam.

REFERENCES

Arai T, Taha H, Mohd-Riduan MN and Mokti SSA (2020). Molecular and morphological evidence for the identity of the giant mottled eel, Anguilla marmorata in Southeast Asia. *Trop. Ecol.* 61: 429-436. DOI: https://doi.org/10.1007/s42965-020-00096-4.

Abdul Kadir SR, Rasid MHFA, Kwong KO, Wong LL, et al. (2017). Occurrence and the ecological implication of a tropical anguillid eel Anguilla marmorata from peninsular Malaysia. *ZooKeys*. 695: 103-110. DOI: 10.3897/zookeys.695.13298.

Arai T (2014). Evidence of local short-distance spawning migration of tropical freshwater eels, and implications for the evolution of freshwater eel migration. *Ecol. Evol.* 4: 3812-3819. DOI: https://doi.org/10.1002/ece3.1245.

Arai T (2016). Taxonomy and Distribution. In: Biology and Ecology of Anguillid Eels (Arai T ed.). *CRC Press*. 1-20. DOI:https://www.crcpress.com/Biology-and-Ecology-of-Anguillid-Eels/Arai/p/book/9781482255157. Downloaded on 6 April 2018.

Arai T, Chin TC, Kwong KO and Siti Azizah MN (2015). Occurrence of the tropical eels, Anguilla bengalensis bengalensis and A. bicolor bicolor in Peninsular Malaysia, Malaysia and implications for the eel taxonomy. *Mar. Biodivers. Rec.* 8: e28. DOI: https://doi.org/10.1017/S1755267215000056.

Arai T and Wong LL (2016). Validation of the occurrence of the tropical eels, Anguilla bengalensis bengalensis and A. bicolor bicolor at Langkawi Island in Peninsular Malaysia, Malaysia. *Trop. Ecol.* 57 (1): 23-31.

Arai T and Chino N (2018). Opportunistic Migration and Habitat Use of the Giant Mottled Eel Anguilla marmorata (Teleostei: Elopomorpha). *Sci Rep.* 8 (5666): 1-10. DOI: https://doi.org/10.1038/s41598-018-24011-z.

Castle PHJ and Williamson GR (1974). On the validity of the freshwater eel species Anguilla ancestralis ege from Celebes. Copeia 2: 569-70.

Ege V (1939). A revision of the genus Anguilla Shaw: a systematic, phylogenitic and geogrephical study. *Dana Rep.* 16: 1-256.

Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*. 39: 783-791.

Hewavitharane CA, Pickering TD, Rico C and Mochioka N (2017). Species identification and morphological differences of Anguillid glass eels recruiting to Viti Levu Island of Fiji in the western South Pacific. *Aquacult. Sci.* 65 (4): 357-366.

Hebert PDN and Barrett RDH (2005). Identifying spiders through DNA barcodes. *Can. J. Zool.* 83: 481-491. DOI: https://doi.org/10.1139/z05-024.

Hebert PDN, Cywinska A, Ball SL and deWaard JR (2003). Biological identifications through DNA barcodes. *Proc. Biol. Sci.* 207 (1512): 313-321. DOI: https://doi.org/10.1098/rspb.2002.2218.

Hall TA (1999). BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symposium Series*. 41: 95-98.

Hoang NV and Duc NH (2012). Research fish fauna species composition of Tam Giang - Cau Hai, Thua Thien Hue Province. *Academia Journal of Biological.* 34 (1): 20-30 (in Vietnamese with English abstract).

Huyen KT and Phu VV (2014). Status of exploitation and management measures and cosnevation development for eel (Anguilla marmorata) resources in Huong river system. *Hue University Journal of Science*. 4/2014: 195-201 (in Vietnamese with English abstract).

Huyen KT and Linh NQ (2019). Characterization of giant mottled eel (Anguilla marmorata) gastrointestinal tract that origin from Thua Thien Hue, Vietnam. *J. Fish Res.* 3(2): 4-9. DOI: 10.35841/fisheries-research.3.2.4-9.

Huyen KT and Linh NQ (2020). Phylogenetic analysis of Anguilla marmorata population in Thua Thien Hue, Vietnam based on the cytochrome C oxidase I (COI) gene fragments. *AMB Expr*. 10: 122. DOI: https://doi.org/10.1186/s13568-020-01059-7.

Linh NQ and Huyen KT (2020). Multiple population structure of the giant eel Anguilla marmorata in Thua Thien Hue, Vietnam base on COI sequences. DOI: https://doi.org/10.21203/rs.3.rs-40345/v1.

Kimura M (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol .Evol.* 15: 111-120.

Kumar R, Singh PJ, Nagpure NS, Kushwaha B, et al. (2007). A non-invasive technique for rapid extraction of DNA from fish scales. Indian *J. Exp. Biol.* 45: 992-997. DOI: https://pdfs.semanticscholar.org/2d3f/616c0f78098c25b74c5e42446d7e20cc586d.pdf.Accessed March 6, 2018.

Kumar S, Stecher G, Li M, Knyaz C, et al. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* 35 (6): 1547-1549. DOI: https://doi.org/10.1093/molbev/msy096.

Kress WJ, Garcia-Robledo C, Uriarte M and Erickson DL (2015). DNA barcodes for ecology, evolution, and conservation. *Trends in Ecology & Evolution* 30: 25-35. DOI: https://doi.org/10.1016/j.tree.2014.10.008.

Larkin MA, Blackshields G, Brown NP, Chenna R, et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*. 23: 2947-2948. DOI: https://doi.org/10.1093/bioinformatics/btm404.

Minegishe Y, Aoyama J, Inoue JG, Miya M, et al. (2005). Molecular phylogeny and evolution of the freshwater eels genus Anguilla based on the whole mitochondrial genome sequences. *Mol. Phylogenet. Evol.* 34: 134-146.

Muchlisin ZA, Batubara AS, Fadli N, Muhammadar AA, et al. (2017). Assessing the species composition of tropical eels (Anguillidae) in Aceh Waters, Indonesia, with DNA barcoding gene cox1. [version 1; peer review: 1 approved, 2 approved with reservations]. *F1000Research.* 6: 258. DOI: https://doi.org/10.12688/f1000research.10715.1.

Nei M and Kumar N (2000). Molecular Evolution and Phylogenetics. Oxford University Press.

Nguyen AT, Tsukamoto K and Lokman PM (2018). Composition and distribution of freshwater eels Anguilla spp. in Vietnam. *Fish Sci.* 84: 987-994. DOI: https://doi.org/10.1007/s12562-018-1239-9.

Palumbi S, Martin A, Romano S, McMillan W, et al. (1991). The simple fool's guide to PCR, Version 2. Department of Zoology and Kewalo Marine Laboratory. Honolulu.

Phu VV and Ha TTC (2008). Biodiversity on species composition of fish fauna in Bu Lu river, Thua Thien Hue province. *Hue University Journal of Science.* 45: 111-121 (in Vietnamese with English abstract).

Phu VV and Dang NT (2008). Biodiversity on Species Composition of the Ischthyofauna in Truoi River, Thua Thien Hue Province. *Journal of Research and Development.* 5 (70): 44-52 (in Vietnamese with English abstract).

Phụng NH (2001). Vietnamese Animals - Episode X. Scientific and Technical Publisher, Hanoi (in Vietnamese).

Watanabe S (2003). Taxonomy of the Freshwater Eels, Genus Anguilla Schrank, 1798. In: Eel Biology (Aida K, Tsukamoto K, Yamauchi K eds). Springer, Tokyo.

Watanabe S, Aoyama J and Tsukamoto K (2004). Reexamnation of Ege's (1939) use of taxonomic characters of the genus Anguilla. Bull. Mar. Sci. - Miami. 74(2): 337-351.

Watanabe S, Aoyama J, Nishida M and Tsukamoto K (2005). A molecular genetic evaluation of the taxonomy of eels of the genus Anguilla (Pisces: Anguilliformes). *Bull. Mar. Sci.* 76(3): 675-690.

Wong LL, Abdul Kadir SR, Adawiah Abdullah RA, Lasuin CA, et al. (2017). Evidence supporting the occurrence and the ecological implication of giant mottled eel, Anguilla marmorata (Actinopterygii: Anguilliformes: Anguillidae), from Sabah, Borneo Island. *Acta Ichthyol. Piscat*. 47 (1): 73-79. DOI: https://doi.org/10.3750/AIEP/02072.

