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OCCURRENCES OF TROPICAL ANGUILLID EELS IDENTIFIED THROUGH DNA BARCODES AT KEDURANG RIVER, SUMATRA ISLAND, INDONESIA

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ABSTRACT

Understanding the geographic distribution of tropical anguillids is important for the conservation of these species. Delimitation of species distribution area is a fundamental task with important implications for the understanding of biodiversity and conservation. However, their geographic ranges are poorly understood, mostly because of the technical difficulties of identifying anguillids by morphological characteristics, especially at the glass eel stage. The current work aims to provide the information on recruitment of tropical anguillid eels in the Kedurang River by using analysis of species identification of an informative DNA segment of *cytochrome oxidase subunit 1*. Results showed that PCR, sequencing and analysis of an informative DNA can be a useful complement to morphological study for more complete biodiversity assessments. The anguillid eels found in Kedurang River were identiûed and further validated as *Anguilla bicolor bicolor*, *A. interioris, A. bengalensis bengalensis*, and *A. marmorata* through glass and adult eels. This study suggests the occurrence of *A. interioris* in the Estuary of Sumatra River would provide the first confirmation for this species in the territory. The information can be useful for understanding the geographic distribution of this species for the establishment and allocation of risk categories to species, both in national protection lists and in those of treaties and international conventions.

Keywords: Anguilla; Sumatra; DNA Barcodes

INTRODUCTION

The freshwater eels of the genus *Anguilla* are catadromous fish with their juvenile growth stage occurring in estuaries, rivers and lakes, and with their spawning area being offshore in the ocean (Tesch, 1977; 2003). The genus is thought to have a tropical origin (Aoyama *et al*., 2001). In contrast to temperate species, tropical anguillids are thought to spawn much closer to their freshwater habitats (Aoyama *et al.*, 2003; Arai, 2014). The signiûcantly shorter migration of tropical anguillid species may result in narrower freshwater distributions than the temperate species, owing to lower dispersal during oceanic larval transportation (Arai, 2020). Moreover, the potential cryptic anguillid species in Indonesian waters has been reported recently (Sugeha, 2010).

In spite of the limited distribution of tropical species, their geographic ranges are poorly understood, mostly because of the technical difficulties of identifying anguillids by morphological characteristics, especially at the glass eel stage (Shirotori *et al.,* 2016). Identification of *Anguilla* at the glass eel stage has various advantages including: 1) glass eels in estuary were in aggregate form which contained all eels species, thus possibility to documenting complete biodiversity was high; 2) it was more practical to collect sample specimens in enough size, eel fish for biodiversity needs; and 3) information that would possibly generated from this stage is very important (e.q. eels recruitment). Mota-Vargas & Rojas-Soto (2012) deûne the distribution area of a species as the fraction of the geographical area where that species is present and can interact in a non-ephemeral manner

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with the ecosystem. Distribution areas are normally recorded and represented by some form of cartographic delimitation in relation to the group of known sites of occurrence of the species, i.e. locality records.

Understanding the geographic distribution of tropical anguillids is important for the conservation of these species. Species distribution area delimitation is a fundamental task with important implications for the understanding of biodiversity and conservation (Lamoureux *et al*., 2006). The restriction of distribution area size is one of the basic criteria for the establishment and allocation of risk categories to species, both in national protection lists (e.g., Junqua *et al*., 1999) and in those of treaties and international conventions (Birdlife International 2000; IUCN 2008). In fact, the aquaculture of freshwater eels relies solely on natural stocks, because artiûcial production of anguillids has not yet been achieved at a commercial scale, and after the collapse of temperate anguillid ûsheries, commercial demands for tropical species have increased rapidly (Crook, 2014). Moreover, the status of tropical anguillids has never been evaluated thoroughly, and even the distributions of each species are poorly known, despite the fact that commercial ûshing of glass eels is already taking place in areas that the species might inhabit (Leander et al., 2013; 2013; Shirotori *et al.,* 2016). To understand the geographic distribution of tropical anguillids for its management and conservation, we analysed anguillid eels from Kedurang River, Sumatra Island, Indonesia. These eels were subjected to identiûcation using an informative mitochondrial *cytochrome oxidase subunit I* (*COI*) sequence analysis. Furthermore, we evaluated the number of species recruited in addition to the importance of the area for a potential conservation area.

MATERIALS AND METHODS

Ethics Statement

A permit to collect fish was given to Arif Wibowo from the Research Institute of Inland Fisheries and Extensions, Ministry of Marine and Fisheries Affairs, Republic of Indonesia. No experimentation was conducted on live specimens during this study, because the permit granted does not extend to experimentation on animals and there are no experimental facilities at the institute.

Area Study, Sample Collection and Preservation

Sampling collections were done at the frontline of Kedurang Estuary, Sumatra Island (Figure 1) and monthly sampling was conducted between February 2017 and March 2018. Sampling and collection of representative samples of glass eels were performed using a modified trap net (Figure 2). Typical of clear, rocky channel, turbulent water of the gravel beds and with rushing current in downstream made Kedurang River is perfect spot for non selective fishing gear (dam/ statistic and relies on the movement of eels) for catching various species of anguillid eel species.

A total of 100 samples were captured during the year at the evening time and all were accumulated in buckets containing clear water. Subsequently, glass eels were kept in technical ethanol (in the composition of 30% mixture: 70% technical ethanol) immediately in the field. After arriving at the Institute's laboratory, the absolute ethanol within each sample was replaced with a new one of absolute ethanol 99%.

Figure1. Trap fishing gear to collect glass eels.

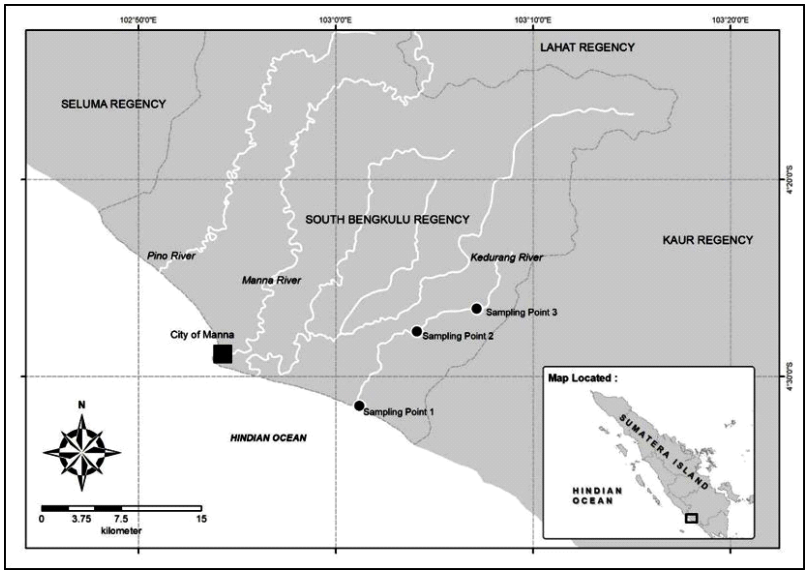

Figure 2. Map showing sampling location of catadromous eels which reflect different habitat associated with current and depth from middle stream to downstream.

DNA Extraction, Amplification and Sequencing

Total genomic DNA was extracted from muscle tissue using a Geneaid extraction procedure as described by company protocol. A partial fragment (530 bp) of the mitochondrial *COI* was amplified using modified universal primers Fish-COI-F and COI-Fish-R, as described by Ivanova et al. (2007). The primer sequences were as follows: Fish-COI-F, 5'-TAA TAC GAC TCA CTA TAG GGT TCT CCA CCA ACC ACA ARG AYA TYGG-3'; COI-Fish-R, 5'-ATT AAC CCT CAC TAA AGG GCA CCT CAG GGT GTC CGA ARA AYC ARAA-3'.

Amplification of the *COI* fragment was performed in a 50-µL reaction volume consisting of 16 µL of ultrapure water, 2 µL of each primer (1 mM), 25 µL of PCR ready mixture solution (KAPPA). The polymerase chain reaction (PCR) cycling parameters included an initial DNA polymerase activation step of 15 min at 95°C, followed by 35 cycles of 30 s at 94°C, 90 s at 55°C and 30 s at 72°C, and ending with a final extension of 5 min at 72°C. The PCR products were visualized on a 1% agarose gel and purified using the PCR purification kit (Thermo Scientific). A sequencing reaction was performed by the EZ-Seq service (Macrogen) using the reverse primer (COI-Fish-R). All sequences were stored in a public domain database, GenBank, the registered sequences attributed to the GenBank (accession numbers MN961249-MN961267; MT155391- MT155483).

Data Analysis

Chromatograms were checked manually and multi sequence alignments were done using MUSCLE (Dereeper *et al*., 2008). Additional sequences were obtained from public database eq. Basic Local Alignment Search Tool (BLAST) searches of the National Center for Biotechnology Information (NCBI) GenBank database. A neighbour-joining tree was constructed with the Kimura 2-parameter (K2P) model using MEGA version 5.0 software (Tamura *et al*., 2013).

RESULTS AND DISCUSSION

Results

A comparison of the DNA sequences with the sequences in BLAST shows at least 4 different kinds of species. However, *Anguilla marmorata* seems to be the dominating glass eel species in the Kedurang River (Figure 3).

The optimal tree with the sum of branch length = 0.17931175 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. This analysis involved 45 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 580 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

Just like the BLAST results, the phylogenetic tree (Figure 4) shows at least 4 different groups. Compassion with the BLAST analysis gives information about the species assignment of each group. Accordingly, group number 1 can be described as *Anguilla marmorata,* group 2 as *A. bengalensis bengalensis*, group 3 as *A. bicolor bicolor* and group 4 can be assigned as *A. interioris.* A phylogenetic tree displays that *A. marmorata* and *A. bengalensis bengalensis are* genetically more similar than individuals from *A. bicolor bicolor* and *A. interioris* population.

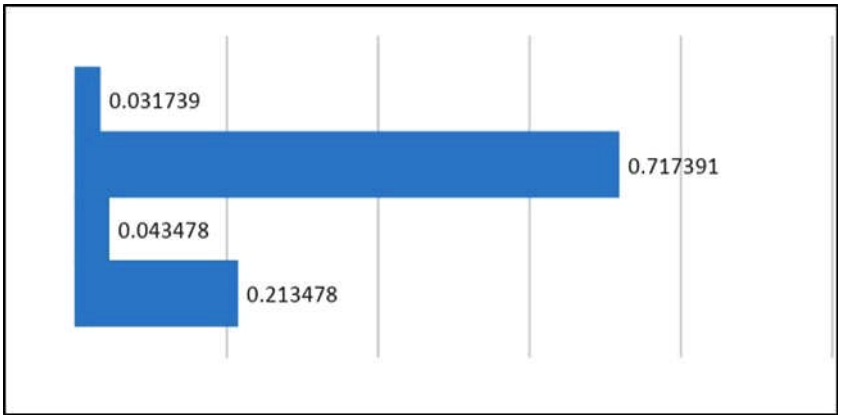

Figure 3. Glass eel composition in the Kedurang river according to the BLAST results.

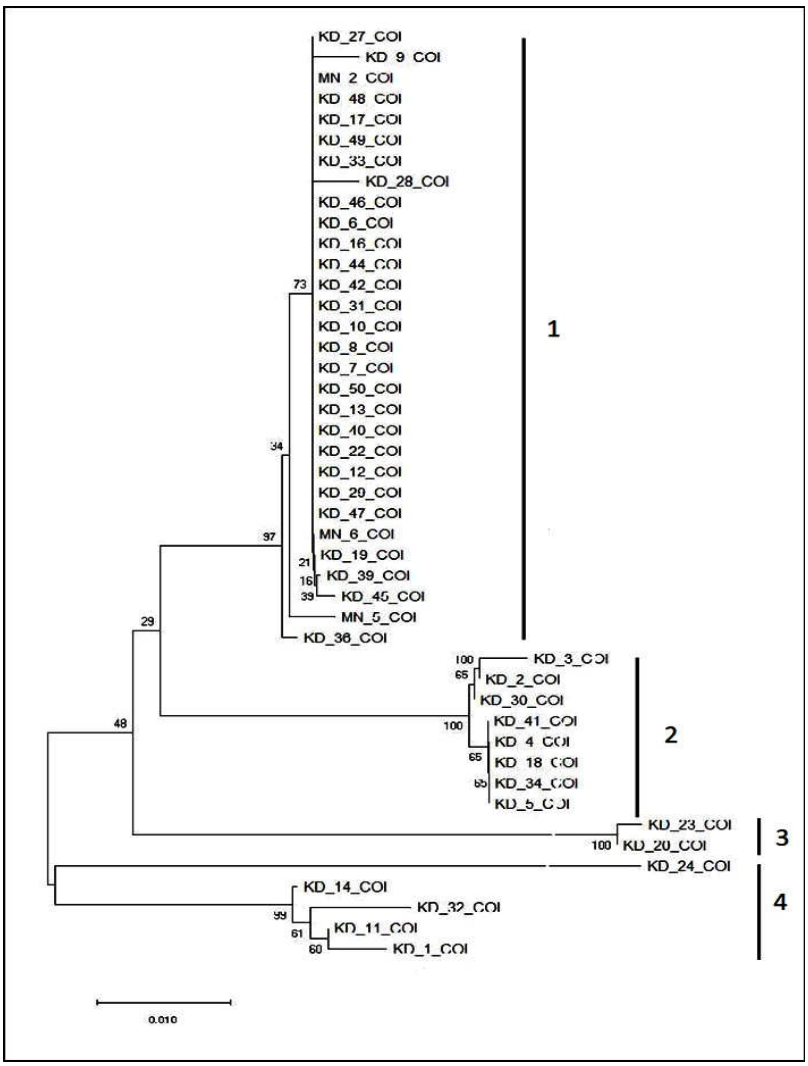

Figure 4. Phylogenetic relationships of glass eels in the Kedurang river. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987).

Discussion

A genetic species is a group of genetically compatible interbreeding natural populations that is genetically isolated from other such groups. Using the genetic data instead of classical morphology is able to estimate >2,000 currently unrecognized species of mammals (Baker & Bradley, 2006). Data that will be useful in identifying these "morphologically cryptic genetic species" are genetic, especially DNA-sequence data. Specimens were considered to belong to a specific taxonomic group only if they formed a cluster with a maximum of 3% (K2P) sequence divergence. This threshold has often been used for specimen identification in different taxonomic groups (e.g. Hebert *et al*., 2003).

For fisheries management, it is crucial to identify these tropical anguillids in order to plan a better conservation strategy, since each one has unique behavioral patterns, and should be independently managed (Muchlisin *et al*., 2017). Molecular phylogenetic research on freshwater eels has revealed that tropical eels are the most basal species originating in the Indonesian and Malaysian regions and that freshwater eels radiated from the tropics to colonize temperate regions (Minegishi et al., 2005). Among the 19 existing eel species and subspecies worldwide, seven occupy the Indonesian rivers. However, the species range distribution of the seven Indonesian species is only an extrapolation of very limited records (Sugeha et al., 2008; Fahmi, 2013).

The International Union for Conservation of Nature (IUCN) has recently listed some tropical species as Endangered or Nearly Threatened (e.g. *A. bengalensis bengalensis* and *A. bicolor* are categorized as Near Threatened (Jacoby *et al*., 2014a), while the status of *A. marmorata* is Least Concern. Moreover, the highlands long-ûnned eel *A. interioris*, has been listed as a "Data Deûcient" (Jacoby et al., 2014b). This is the first description of the occurrence and distribution of four species of *Anguilla* (*A. bicolor bicolor*, *A. interioris A. bengalensis bengalensis*, and *A. marmorata)* within estuary in Sumatra Island as identified through both analysis of glass and adult eels by genetic analyses. In previous studies, three eels, i.e., *A. bengalensis bengalensis*, *A. marmorata*, and *A. bicolor bicolor*, occurred in Peninsular Malaysia (Arai & Abdul Kadir, 2017; Abdul Kadir *et al*., 2017). This is indicatory of DNA barcoding being successful in identifying species of eels in Kedurang River that cannot be identified by morphological data. The study indicates that multiple species of glass eels migrate from the sea into freshwater.

Anguilla bengalensis bengalensis is widely distributed in Sri Lanka, Bangladesh, India, Myanmar, Sumatra Island in Indonesia, and the Andaman Islands (Watanabe *et al*., 2004). In previous studies, *Anguilla marmorata* was reported to exist in Sumatra waters (Muchlisin et al., 2017; Fahmi, 2013). In fact, the difûculty in distinguishing both *Anguilla marmorata* and *Anguilla bengalensis* is augmented by their overlapping morphological characteristics, which cause further identiûcation ambiguities (Arai & Wong, 2016). The FDI (fin difference index) values in the long-ûnned eels (both species) were 11–12 and could not be used to identify samples morphologically due to the overlapping geographical distribution of several eel species and proximity in the tropical region (Arai *et al*., 2015).

Anguilla bicolor has the second widest geographic distribution of any species of the genus *Anguilla*, except for *Anguilla marmorata* (Arai *et al*., 2015; Arai & Taha, 2021). *Anguilla bicolor* is distributed from the eastern coast of Africa, through the Indonesian seas to New Guinea, adjacent to the Paciûc Ocean (Ege, 1939). Recently, Fahmi (2013) and Muchlisin *et al*. (2017) found a new distribution range of *Anguilla bicolor bicolor* in Sumatra waters and the present study also supports the occurrence of *Anguilla bicolor* in Kedurang River by means of molecular genetic signatures.

Anguilla interioris is endemic, limited to Indonesia with limited information available on the distribution of this species but currently known only from Sumatra waters and the northern half of New Guinea (Fahmi, 2013). In the previous studies, *Anguilla interioris* was not reported to exist in Aceh waters (Muchlisin *et al*., 2017). However, larvae were found in the Indonesian seas around Central Sulawesi, the western Central Pacific and the eastern Indian Ocean (Aoyama *et al*., 2007). Therefore, several anguillid eels have multiple spawning sites and been divided into continental subpopulations with a wider distribution than presently thought (various authors in Jacoby & Gollock, 2014; Watanabe *et al*., 2004; Arai, 2020). *Anguilla interioris* is classified as Data Deficient by the IUCN in 2014. Its population status and trend are unknown, due to the lack of information; however, it has quite specific habitat preferences and may be susceptible to overexploitation, particularly if demand shifts to this species (UNEP-WCMC 2015).

This study highlights the occurrence of four species of *Anguilla* (*A. bicolor bicolor*, *A. interioris, A. bengalensis bengalensis*, and *A. marmorata)* within the estuary in Sumatra Island. Although those species

are widely distributed, little information is available on the study of the life story and migration patterns of this species (Arai *et al*., 2015; Arai, 2020; Arai & Taha, 2021). This study suggests the occurrence of *Anguilla interioris* in the estuary of Sumatra River, providing the first confirmation for this species in the territory. The information can be useful for understanding the geographic distribution of this species for the establishment and allocation of risk categories to species, both in national protection lists and in those of treaties and international conventions. Further studies regarding migration pattern, biology and ecology of tropical anguillid eels within this river are needed to understand their mysterious life history for better management and conservation strategy.

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