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Phylogenetic and Genetic Diversity of the Spiny Eel *Mastacembelus unicolor* (Mastacembelidae) from Kediri, East Java, Indonesia

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Abstract: The spiny eel from the family Mastacembelidae are potential fish for aquaculture because it's high economic value as an ornamental and as a consumption fish. Unfortunately, this fish have not been cultivated yet and up to present local people depend on wild-caught. This situation leads to severe population decline of this species in nature. Efforts to cultivate or protect this species in nature are therefore crucial. Species identification is the first and essential step performing domestication or conservation of a species. In this study, we conducted morphological identification as well as phylogenetic and genetic diversity analyses using cytochrom c oxidase subunit I gene. The results revealed spiny eel from Kediri can be identified as *Mastacembelus unicolor* based on both morphological and molecular analyses. The phylogenetic analyses suggested that individuals from Kediri have the closest relationship to *M. unicolor* individuals from West Java. Individuals sequenced from Kediri exhibit identical haplotype which indicating no genetic polymorphism between individuals. The individuals of Mastacembelidae in the Kediri were morphologically and genetically identified as *Mastacembelus unicolor*. The genetic analysis result shows Kediri have 53.13% AT, 48.67% GC, and one haplotype with low hd and low π value.

Keywords: COI; Genetic diversity; Identification; *Mastacembelus*; Phylogenetics

Introduction

The spiny eel Mastacembelidae is family that has vast distribution in tropical and subtropical areas of Africa, the Middle East, South Asia, and Southeast Asia (Aly et al., 2023). The family Mastacembelidae consists of 3 genera: *Macrognathus*, *Mastacembelus*, and *Sinobdella* (Gholamhosseini et al., 2022; Parenti et al., 1995). In Indonesian waters, there are nine species distributed from this family, five species from the *Macrognathus*, and four species from *Mastacembelus*. In Java Island, two genera from the Mastacembelidae family *Macrognathus* and *Mastacembelus* are recorded: *Macrognathus aculeatus* and *Mastacembelus unicolor* (Kusuma et al., 2023; Murdy et al., 1994). In this island, the two species from Mastacembelidae have been reported in the Brantas River, Bengawan Solo River (Rahma, 2021), Cimanuk River (Sentosa et al., 2011), and Cilutung River (Dahruddin et al., 2017). Specifically in Brantas River, both *Macrognathus aculeatus* and *Mastacembelus unicolor* can be found.

Kediri is a city crossed by Brantas River. Fishes from *Mastacembelidae* in this city important commodity as they have high economic value as an ornamental as well as consumption for local people. Thus, these fishes have the potential to be cultivated. However, to meet the need for these fishes, local people still rely on wild-caught. Efforts to cultivate this fish must be immediately

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started. Species identification is the first step of research on systematic biology and biodiversity (Duong et al., 2020), genetic information is key for developing aquatic resources management and crucial step in conducting domestication, aquaculture and conservation (Pramono et al., 2019). Species determination between *Macrognathus aculeatus* and *Mastacembelus unicolor* can sometimes be difficult, especially for those who do not have knowledge in species identification. Therefore, accurate and rapid identification using molecular marker such as DNA barcode region of cytochrome c oxidase subunit I (COI) is essential to complete morphological identification. The COI gene is known to have many advantages, such as have few deletions and insertions in its sequence and conserved so that it can be used as a barcode or DNA identifier for each species (Anjarsari et al., 2021; Hebert et al., 2003; Meilana et al., 2016). In this study, we conducted morphological dan molecular identification of *Mastacembelidae* individuals collected in Kediri, East Java, Indonesia. We then performed phylogenetic analysis to determine the phylogenetic position of this species among several Mastacembelidae species. We also estimated the genetic diversity and compared it with other sequence data from GenBank.

Method

Specimen Collection

A total of five samples (Kediri 1 – Kediri 5) were collected from the Brantas River, Kediri, East Java (Figure 2). All individuals were identified morphologically based on Murdy et al. (1994), after the morphological identification was complete, the samples were preserved using 96% alcohol. All individuals were deposited at Specimen Depository, Faculty of Fisheries and Marine Science, Universitas Brawijaya as UB.1.692.1–UB.1.692.5. *M. unicolor* sequences from two locations (West Java, Indonesia, and Malaysia) were added from Genbank for genetic comparison. To identify family relationships, sequences of *M. erythrotaenia*, and *M. notophthalmus* were added. This addition is based on the distribution of Mastacembelidae especially *Mastacembelus* in Indonesia (Kottelat et al., 1993). In Indonesia, there are three species of *Mastacembelus* (*M. unicolor*, *M. erythrotaenia*, and *M. notophthalmus)*. *Sinobdella sinensis* as outgroup based on family similarities (Mastacembelidae). This study used sequence *M. unicolor* from West Java KU692615 – KU692619; *M. unicolor* from Malaysia KT944641, JQ768975 – JQ768978; *M. erythrotaenia* KT944598 – KT944599; *M. notophthalmus* is KT944621 – KT944625 and *Sinobdella sinensis* MK321934 – MK321935 for outgroup.

DNA Extraction, Amplification and Sequencing

DNA was extracted from the fins using the DNA Extraction Kit, Promega. The COI gene was amplified using primers FishF1: 5-TCAACCAACCA CAAAGACATTGGCAC-3C, FishR1: 5-TAGACTTCTGGGGTGGCC AAAGAATCA-3A (Ward et al. 2005). PCR amplification uses a predenaturation temperature 95°C for 2 minutes, denaturation 94°C for 40 seconds, annealing 54°C for 40 seconds, extension 72°C for 1 minute 10 seconds, and final extension temperature 72°C for 10 minutes. The PCR product was visualized by 1.5% agarose gel electrophoresis and sequenced using the sanger sequencing method.

Data Analysis

Partial COI genes from five samples were edited using Chromas software v2.6.6 to cut noise in the electrophorogram, then a consensus was made using the UGENE v49.0. Sequences were combined with sequences from Genbank and then aligned using Mesquite v.3.8 (Tapia et al., 2017). Genetic diversity including number of haplotype (*h*), haplotype diversity (*hd*), and nucleotide diversity (π) was analyzed using the DnaSP v5.10.1. Phylogenetic analysis was performed in MEGA11 using maximum likelihood method (Tamura et al., 2013). Kimura-2 parameter was selected as the best substitution method based on Akaike Information Criterion. The maximum likelihood method was chosen using bootstraps with 1000x replication.

Figure 1. Research scheme

Result and Discussion

Morphological analysis

Morphological identification obtained Kediri 1-Kediri 5 has an elongated body and rounded caudal fin, has spines along the dorsal fin, there are short spines in front of the anal fin, does not have a ventral fin but has a snout, the tail fin is slightly separated from the dorsal and anal fins and has a rounded color pattern without any red lines. Brantas population has D XXXII-XXXIII, 79-80 A; III, 73-79; C 18-20. Kottelat et al. (1993) described the morphology of *M. unicolor* has D XXXIV-XXXV, 79-90; A III,73-86; C 19-21, has a caudal fin that is slightly separated from the dorsal and anal fins with no red stripes. The morphological characters have many similarities with *M. unicolor* so Kediri belongs to the *M.*

unicolor species, but there are deviations in the dorsal fin spines. Yunus et al. (2018) also explained that *M. unicolor* can be characterized by a round yellow color pattern on its body, the color pattern extends parallel from behind the operculum to the base, the caudal fin is separate from the dorsal and anal fins, and has sharp spines on the front of the dorsal fin.

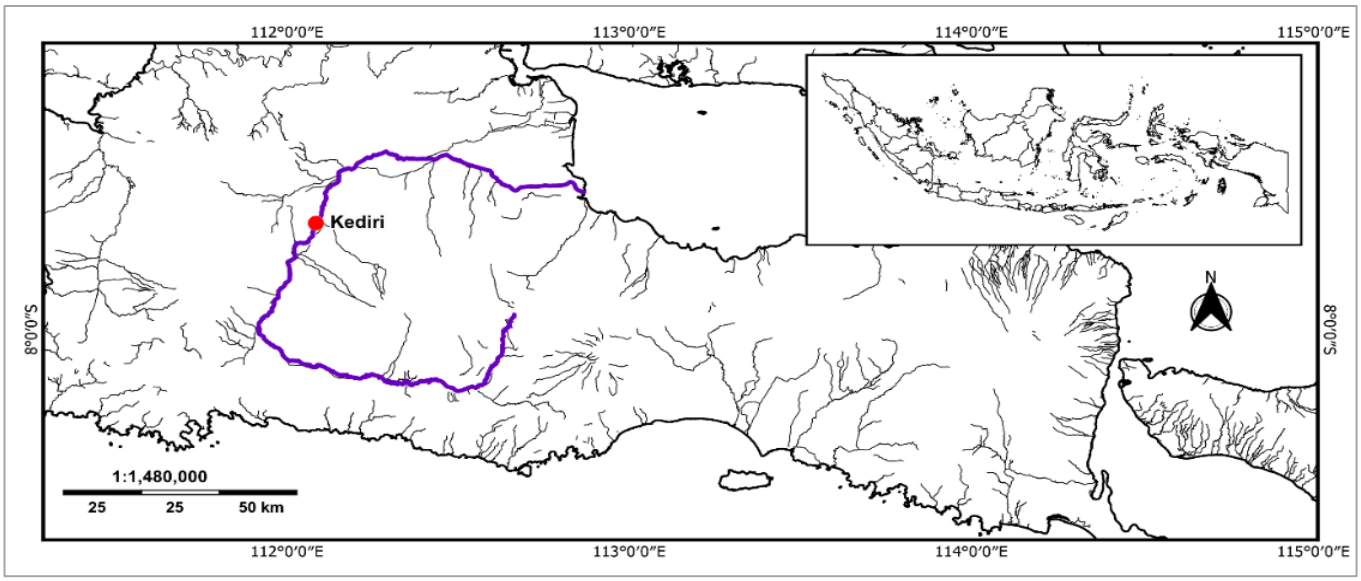

Figure 2. A map shows sampling locality of *M. unicolor* in Kediri, the line with purple color indicates Brantas River

Figure 3. *M. unicolor* fish from Brantas River, Kediri, East Java

Table 1. Nucleotide Composition of *M. unicolor* Fish

Group	Location	Number of individuals	% Nucleotide C	T	A	G
1	Kediri	5	29.31	27.02	26.11	17.56
2	West java	5	29.95	26.34	26.2	17.51
3	Malaysia	5	29.14	26.99	26.38	17.48
Average			29.31	27.02	26.11	17.56

Table 2. Number of Haplotype, Haplotype Diversity, and Nucleotide Diversity M. unicolor Fish

Location	Number of haplotype (*h*)	Haplotype diversity (*hd*)	Nucleotide diversity (π)
Kediri	1	0.00	0.00
West java	2	0.400	0.007
Malaysia	1	0.00	0.00

Table 3. Genetic Distance between Populations

	Kediri	West java	Malaysia	*M. erythrotaenia*	*M. notophthalmus*
Kediri					
West Java	0.0068				
Malaysia	0.0127	0.0124			
M. erythrotaenia	0.0054	0.0095	0.0120		
M. notophthalmus	0.0960	0.0903	0.0887	0.0914	
Sinobdella sinensis	0.1709	0.1781	0.1687	0.1707	0.1887

Genetic Analysis

The COI mtDNA sequence from 15 sequences (five from this study and ten from Genbank) has a total length of 655 bp. The sequences have average percentages of C, T, A, and G of 29.31%, 27.02%, 26.11%, and 17.56%, the percentage of nucleotide for each population shown in Table 1. The nucleotide composition of three populations contains the adenine and thymine pair (A+T) which is more dominant than the guanine and cytosine pair (G+C). The percentage of A+T (AT%) is highest in Malaysia (53.37%), then Kediri (53.13%), and West Java (52.54%). Ahmed et al. (2020) explains that composition of nucleotide bases in the genus *Mastacembelus* is C (25.6%), T (22.4%), A (33%), and G (19.5%) with GC (45.1%) and AT (55.4%) values with the COI sequence nucleotide bases having a higher AT% content than GC% content, similar to the pattern observed in Australia (Ward et al., 2005) and Canada (Steinke et al., 2009). One of the characteristics of COI-encoding genes is that they have a lower guanine composition than cytosine composition. The high G+C composition can protect DNA from heat-induced degradation, as the G-C bond is a strong bond, when compared to A-T (Gu and Li, 1994; Kusuma et al., 2021). The percentage composition of the G+C pair (GC%) is opposite of the A+T pair. The nucleotide percentage of the gene varies depending on the organism, eucariotic genome has 30 - 35% adenine, 30 - 35% thymine, 15 -20% guanine, and 15 -20% cytosine. In general, fish genomes tend to have relatively balanced AT% and GC%, although GC% is less than AT% (Lamadi, 2023; Slocombe et al., 2021; Symonová et al., 2019).

Table 4. Genetic Distance within Populations

Populations	d
Kediri	0.0000
West java	0.0085
Malaysia	0.0000
M. erythrotaenia	0.0015
M. notophthalmus	0.0140
Sinobdella sinensis	0.0018

The number of haplotypes (*h*) obtained four haplotypes with the highest haplotype diversity (*hd*) in West Java, and the lowest in Kediri and Malaysia. The highest nucleotide diversity (π) value is West Java, the lowest in Kediri and Malaysia. Genetic variation is shown in Table 2. Kediri and Malaysia have hd=0.00 and π=0.00 and West Java has hd=0.4 and π=0.007 Kediri and Malaysia are included in Category One, which indicates that a bottleneck effect has occurred, whereas West Java is included in Category Three, which indicates that the population is starting to stabilize. Grant et al. (1998) explain that there are four categories to see the comparison between Hd and π. The first category is low *hd* and low π (*hd* < 0.5 and π < 0.5%); the second category is high *hd* and low π (Hd ≥ 0.5 and π < 0.5%); the third category is low *hd* and high π (Hd < 0.5 and π ≥ 0.5%); and the fourth category is high *hd* and high π (Hd ≥ 0.5 and π ≥ 0.5%). The first category indicates that a population has occurred founder effect or bottleneck effect. The second category indicates that the small population from the bottleneck effect has rapid population expansion or growth and experienced many mutations. The third category characterizes populations that are beginning to stabilize, and have several different haplotypes due to secondary contact between isolated populations or because of strong barriers to previously large and stable populations. The fourth category indicates that the population has stabilized after a long evolutionary history.

The low genetic variation Kediri and Malaysia can be caused by the bottleneck effect. The bottleneck effect in Kediri could probably be due to anthropogenic activities including overfishing, habitat degradation, and water pollution. Overfishing and habitat loss have had a negative impact on population decline and genetic diversity, and this may be the cause of the low genetic variation (Thirumaraiselvi et al., 2015). The value of genetic diversity is generally influenced by gene mutation, population size, reproduction, migration/distribution, and natural selection (Chiu et al., 2013). Genetic variation can occur as a result of the response to selection and adaptation patterns of organisms to environmental changes (Prehadi et al., 2014). Low haplotype and nucleotide diversity is an indication of low genetic diversity (Triandiza et al., 2021). The higher the value of haplotype diversity (Hd), the higher the level of genetic diversity of a population, and vice versa, if the value of haplotype diversity is low, the level of genetic diversity of the population is also low (Akbar et al., 2014). In general, genetic variation is affected by mutation, natural selection, mating, environmental changes, and population size. Large populations tend to have high genetic diversity because they can interbreed randomly so they can maintain genetic diversity. Migrate fish also tend to have a high level of genetic diversity because the chance of crossing with other populations is higher (Ath-thar et al., 2017). Populations with wider habitats tend to have higher genetic diversity than populations with narrower habitats (Hendiari et al., 2020).

The low value of genetic diversity in a species in the long run will have several negative impacts, such as decreased genetic variation and higher homozygotes (Francis et al., 2010; Frankham, 1997). Some populations that have a high value of genetic diversity generally have a high level of adaptation due to the variation in genotypes that appear in response to changes in environmental conditions (Thomsen et al., 2015). The

value of genetic diversity will affect several aspects including disease resistance, response to environmental changes, behavior, and productivity/biomass. Genetic diversity variables also depend on the population (Nuryanto et al., 2017). In conservation and selective breeding efforts, potential parents are those with high genetic diversity (Ukenye et al., 2019). Large populations will tend to have greater genetic diversity than small populations, with this correlation, estimating the population size of species can be done. Genetic diversity data is needed as a first step in efforts to conserve populations in nature. Genetic diversity can be used to estimate population size and adaptability. The higher the genetic diversity, the larger the population size, and the better the adaptability of species in the population. A population with more diverse genetics is more resistant to disturbances such as extreme environmental conditions (Ketchum et al., 2016).

Phylogenetic Analysis

The *M. unicolor* sequence was reconstructed using a phylogenetic tree by adding the *M. erythrotaenia* and *M. notophthalmus* sequences from Genbank to determine the relationship between the species (Figure 3). Genetic distance is also calculated to determine differences between and within populations. The phylogenetic tree shows a 0.02 scale, which can be interpreted as a genetic difference of 2%. The Kediri and Malaysia have a distance within populations of 0.0% so there are no genetic differences within each location, whereas in West Java, there is a difference within the population is 0.85%, in *M. erythrotaenia*, 0.15%, in *M. notophthalmus* 1.4 % and in *S. sinensis* 0.18% (Table 4).

Genetic distance between populations shows Kediri has a distance of 0.68% with West Java, 1.27% with Malaysia, 0.54% with *M. erythrotaenia*, 9.6% with *M. notophthalmus*, and 17.09% with *S. sinensis* (Table 3). The genetic distance of Kediri, West Java, Malaysia, and *M. erythrotaenia* have a genetic distance <2% so they belong to one species. Similar fish species are characterized by a p-distance value of <2% (Bingpeng et al., 2018), so p-distance value >2% is generally categorized as a different species. Greater genetic distance shows that there are many differences between each individual and vice versa.

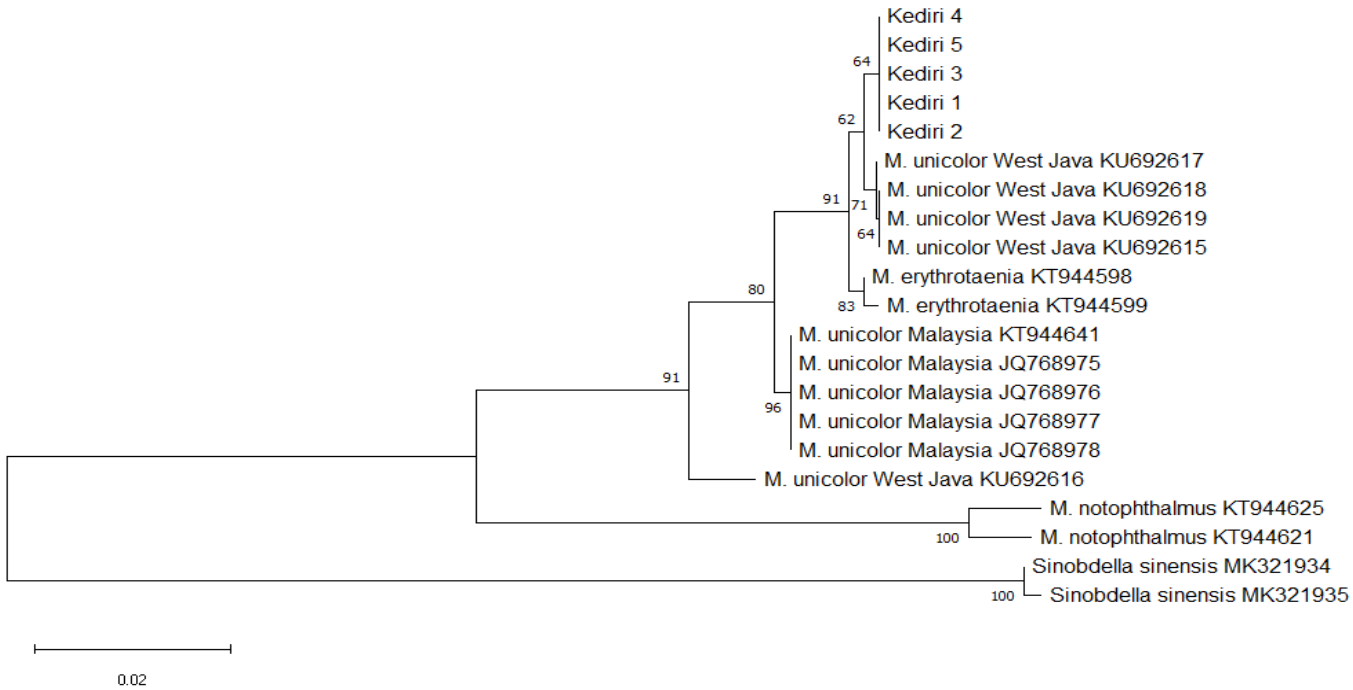

Figure 4. A maximum likelihood tree constructed from 655bp of COI gene sequence

The phylogenetic tree shows that Kediri has closest relationship with *M. unicolor* in West Java, Indonesia. The result based on genetic distance and phylogenetics, Kediri can be described as *M. unicolor* species. The presence of *M. erythrotaenia* in the middle *M. unicolor* is suspected there has been misidentification of the species. Duong et al. (2020), explain that the spiny eel family Mastacembelidae has been nominally classified into 141 species but has undergone several revisions and currently there are 88 valid species recognized. The many differences between nominal and valid species imply that the identification and classification of species in this family is very complex. Traditionally, spiny eel species are identified based on measurable traits such as dorsal fin spines, color, spots, stripes, or reticulate patterns on the body. However, these key characteristics

can vary between life stages or overlap between species, easily leading to misidentification of species. Determining fish species based on a phylogenetic approach taking into account evolutionary levels is very important, especially for taxa whose classification categories are still debated. Simbolon et al. (2021) explained that the use of the COI gene combined with phylogenetic tree analysis along with genetic distances is known to be able to identify and study diversity and evolutionary history. Compiling a phylogenetic classification is the best way to compile biodiversity information and has become a universal practice in biological classification (Fietri et al., 2021). Phylogenetic tree reconstruction can provide information regarding genetic relationships between species within one population and between populations (Saleky et al., 2021).

Conclusion

The individuals of Mastacembelidae in the Kediri were morphologically and genetically identified as *Mastacembelus unicolor*. The genetic analysis result shows Kediri have 53.13% AT, 48.67% GC, and one haplotype with low *hd* and low π value. Kediri is included in category one which indicates a bottleneck effect. The bottleneck effect in the Brantas population could probably be due to anthropogenic activities including overfishing, habitat degradation, and water pollution. Phylogenetic analysis suggested that the individuals from Kediri have the closest relationship to the individuals from West Java. Further research on this species is needed, especially by taking individuals at different locations with a larger number of samples to describe more broadly the genetic diversity and relationships of *M. unicolor* in the Brantas River.

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Author Contributions
The authors in this research are divided into executor and advisor. Conceptualization IS, MSW, AY, WEK; methodology IS, BFHAL; software IS, BFHAL; validation IS, MSW, AY, WEK; analysis IS, MSW, AY, BFHAL, WEK. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest
The author declares no conflict of interest in this research.

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