Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(6): 657 – 671 (2025)
www.ejabf.journals.ekb.eg

Population Genetics Analysis of *M. gulio* in Three Rivers in Pasuruan, East Java Using COI

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ARTICLE INFO

Article History:
Received: Aug. 30, 2025
Accepted: Nov. 1st, 2025
Online: Nov. 15, 2025

Keywords:
Bioindicator,
Diversity,
Euryhaline,
Pollution,
Polymorphism

ABSTRACT

The study of aquatic environments can be conducted through the organisms that inhabit them, one of which involves a population genetics approach. Population genetics has been widely applied to various taxa, including fish; however, this approach remains very limited for keting catfish (*Mystus gulio*), especially in Pasuruan. Previous research has examined the environment and morphology of *M. gulio* in three Pasuruan rivers—Rejoso, Welang, and Gembong—but no population genetics study of *M. gulio* in Pasuruan and the surrounding areas has been conducted. Genetic analysis using the mitochondrial DNA (mtDNA) region of cytochrome oxidase subunit I (COI) can support molecular identification, as well as the estimation of genetic diversity and population structure. Sampling was carried out in three rivers in Pasuruan; the fish were preserved, and DNA extraction was performed up to the sequencing stage. Sequencing results were analyzed using BLAST identification, haplotype network analysis, phylogenetic trees, genetic p-distances, and pairwise Fst. The genetic diversity of *M. gulio* in each river in Pasuruan was low, with a nucleotide diversity (Pi) value of 0.00068 and a haplotype diversity (Hd) value of 0.400. Misidentifications in GenBank based on BLAST results for the Cilacap population were corrected using the haplotype network and phylogenetic tree generated in this study. The populations from each river are likely still connected, including those in Cilacap, as indicated by an Fst value of 0. It is possible that *M. gulio* migrates via the sea, as suggested by the low genetic distance (1.7%) between the Indonesian and Indian populations. Several overlaps were observed, which may represent a taxonomic conundrum.

INTRODUCTION

The number of Bagridae species capable of living in brackish water is quite limited, while habitat overlap with their close relatives in the Ariidae family creates potential for misidentification, including the keting catfish (*Mystus gulio*). Bagridae is a family within the order Siluriformes, widely distributed from Africa to Asia (Old World). Members of this family share morphological similarities with Pimelodidae, but the families are distinct in their distributions (**Nelson *et al.*, 2016; Rais *et al.*, 2022**). Of approximately 240 Bagridae species worldwide, most of them do not inhabit saline environments; therefore, their distribution generally depends on paleogeography (**Yang *et al.*, 2009**), and they were previously thought to occupy only freshwater habitats (**Nelson *et al.*, 2016**). Only a small number of Bagridae species—around 12—are capable of living in brackish water, including *Mystus wolffii* (**Ng, 2012; Rais *et al.*, 2017**), *M. gulio* (**Kumar *et al.*, 2019; Paujiah *et al.*, 2023**), and *Hemibagrus nemurus* (**Kurniawan *et al.*, 2022**). About 62 Bagridae species occur in the Sundaland region, but only around five are distributed in Java: *Hemibagrus planiceps* (**Dodson & Lecomte 2015; Syaifudin *et al.*, 2017**), *H. nemurus* (**Syaifudin *et al.*, 2017; Kurniawan *et al.*, 2022**), *M. singaringan* (**Budhi *et al.*, 2019; Pramono *et al.*, 2019**), *M. gulio* (**Paujiah *et al.*, 2023; Ayuningrum *et al.*, 2025**), and *M. nigriceps* (**Pertiwi *et al.*, 2025**).

Several species within Bagridae and Ariidae are commonly referred to as keting catfish, including *Arius* spp. (**Marsyalita *et al.*, 2012**), *M. nigriceps* and *M. gulio* (**Lestariaji *et al.*, 2025; Ayuningrum *et al.*, 2025**), highlighting the high morphological similarity between the two families. The Ariidae family inhabits saline environments such as estuaries and coastal waters (**Nelson *et al.*, 2016**), allowing some Bagridae members to co-exist with Ariidae species. The taxonomic issues surrounding *M. gulio* span multiple levels—from family to species—due to morphological similarity, overlapping distributions, and population-level uncertainties.

M. gulio has a wide distribution, but the range of each population must be assessed to understand anthropogenic impacts on this species, including population structure and overall health. This species is distributed broadly from South Asia (West Indoburman) to Southeast Asia (East Indoburman) (**Kumar *et al.*, 2019**). Such an extensive distribution is uncommon among Bagridae or other freshwater fishes (**Conte-Grand *et al.*, 2017**), making the distribution pattern and population boundaries of *M. gulio* poorly defined. Unclear population ranges hinder conservation efforts (**Chua *et al.*, 2019**). Furthermore, assessment of anthropogenic impacts on freshwater ecosystems remains limited (**Antonelli *et al.*, 2023**). Anthropogenic stressors interact with natural ones, amplifying impacts through pollution, salinization, sedimentation, fragmentation, pesticides, invasive species, and more (**Bănăduc *et al.*, 2024**). Similar impacts occur in saline ecosystems, such as estuarine and marine habitats, exemplified by acidification (**Cai *et al.*, 2021**). Environmental stress across aquatic ecosystems affects population health, and genetic variation analyses can be used to estimate overall population conditions (**Lamadi *et al.*, 2023**). Studies assessing anthropogenic impacts through population genetic diversity in *M. gulio* from rivers in Pasuruan, East Java haven't yet been conducted, although morphological and growth analyses have been performed (**Lestariaji *et al.*, 2025**).

Genetic analyses for species identification and population-level studies are widely used. One of the most common markers is the cytochrome oxidase subunit I (COI) region due to the maternal inheritance of mitochondrial DNA (**Kim *et al.*, 2022**). Species confirmation can be carried out using the basic local alignment search tool (BLAST) on NCBI (**Wiadnya *et al.*, 2023**), followed by additional analyses such as phylogenetic trees, haplotype networks, and genetic distances (**Lamadi *et al.*, 2023; Paricahya *et al.*, 2024a**). Population-level studies may also incorporate genetic variation to assess population health and use pairwise Fst analyses to estimate gene flow and population structure (**Behera *et al.*, 2017**). Studies of *M. gulio* using these approaches are urgently needed to identify potential threats and guide management. This study was conducted to confirm species identity and estimate population health of *M. gulio* based on COI genetic information from three populations in Pasuruan.

MATERIALS AND METHODS

Time and Location of Research

Specimens of *M. gulio* were collected from three different rivers in Pasuruan, namely the Gembong River, Rejoso River, and Welang River (Fig. 1). The study was conducted from August to October 2024. Specimens of *M. gulio* were caught and collected alive from freshwater streams by local hunter using fishing rod, then transferred to Brawijaya University Laboratory. Specimens were euthanized and preserved using periodic cold temperatures, then conditioned for tissue collection.

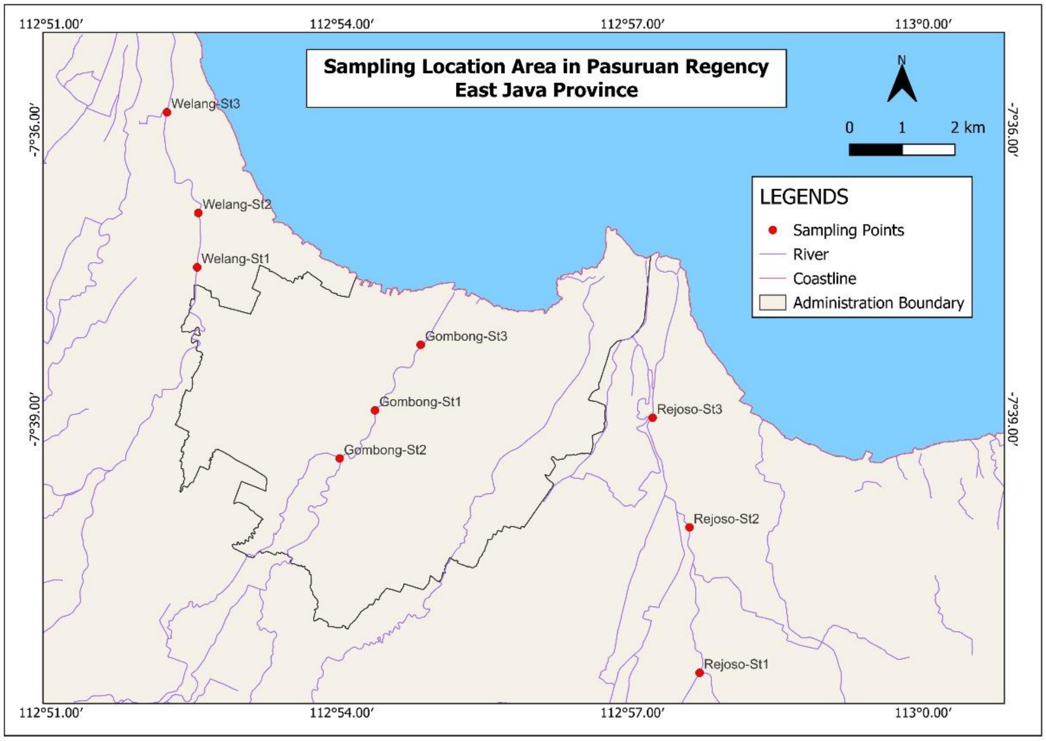

Fig. 1. Map of the study areas in Pasuruan at Welang, Gembong, and Rejoso rivers

Research Methods

Molecular Analysis

Five individuals from each river were taken for genetic testing, resulting in a total of 15 individuals of *M. cf. gulio*, each with approximately 20 mg of muscle tissue (**Lamadi *et al.*, 2023**). DNA extraction was performed using the Wizard® Genomic DNA Purification Kit and amplified using primer F1 Universal for Fish (5′TCAACCAACCACAAAGACATTGGCAC3′) and primer R1 Universal for Fish (5′TAGACTTCTGGGTGGCCAAAGAATCA3′) (**Ward, 2012**).

Amplification was carried out in two stages: the first stage used separate primers (PCR mix 15µL) in five initial cycles with an annealing temperature of 55°C; the second stage incorporated both primers (total PCR mix 30µL) in 30 cycles with an annealing temperature of 65°C. The denaturation and extension temperatures for both stages were 96 and 72°C, respectively.

The amplicons were quantitatively assessed and sent to Apical Scientific Sdn Bhd, Malaysia. Sequences were analyzed using Chromas 2.6.6, UGENE 46.0, Mesquite 3.70, DnaSP 6, Network 10.2, and MEGA 11 (**Paricahya *et al.*, 2024b**).

RESULTS AND DISCUSSION

Genetic Analysis Result

The results of further genetic analyses confirmed that the keting catfish from the three rivers were *M. gulio*. Molecular identification using the basic local alignment search tool (BLAST) feature on the National Center for Biotechnology Information (NCBI) showed that the sequences aligned more closely with *Arius subrostratus* than with *M. gulio* based on percent identity (Per. Ident). The Per. Ident values for the sequences in this study reached 100% for *A. subrostratus*, while the highest value for *M. gulio* was 99.09%. However, the Query Cover values were higher for *M. gulio* (100%) than for *A. subrostratus* (99%). Further analyses using phylogenetic trees and haplotype networks indicated that the *A. subrostratus* sequences in NCBI were misidentified. These misidentified sequences originated from the Cilacap population.

Phylogenetic analyses and haplotype networks showed the relative affinities of *M. cf. gulio* from Pasuruan with various taxa, both intrafamily and interfamily (Fig. 2). Previous genetic identifications had placed *M. gulio* within the Bagridae family and *A. subrostratus* within the Ariidae family. Based on partial COI analysis, the two families are separated by Pangasidae (represented in the dataset by *Pseudolais micronemus* from NCBI), making the distinction between them phylogenetically clear, even though the haplotype network does not resolve this separation as distinctly.

Within Bagridae, the phylogenetic tree clearly separates the genera *Mystus* and *Hemibagrus*. However, the haplotype network shows different patterns. The *M. gulio* clade is separated from the *M. singaringan* clade by at least 138 bp, from the *Hemibagrus nemurus* clade by at least 99 bp, and the *M. singaringan* clade is separated from the *H. nemurus* clade by at least 102 bp. The closer genetic affinity between *Mystus* and *Hemibagrus* compared with relationships within *Mystus* itself may contribute to ambiguity in their classification.

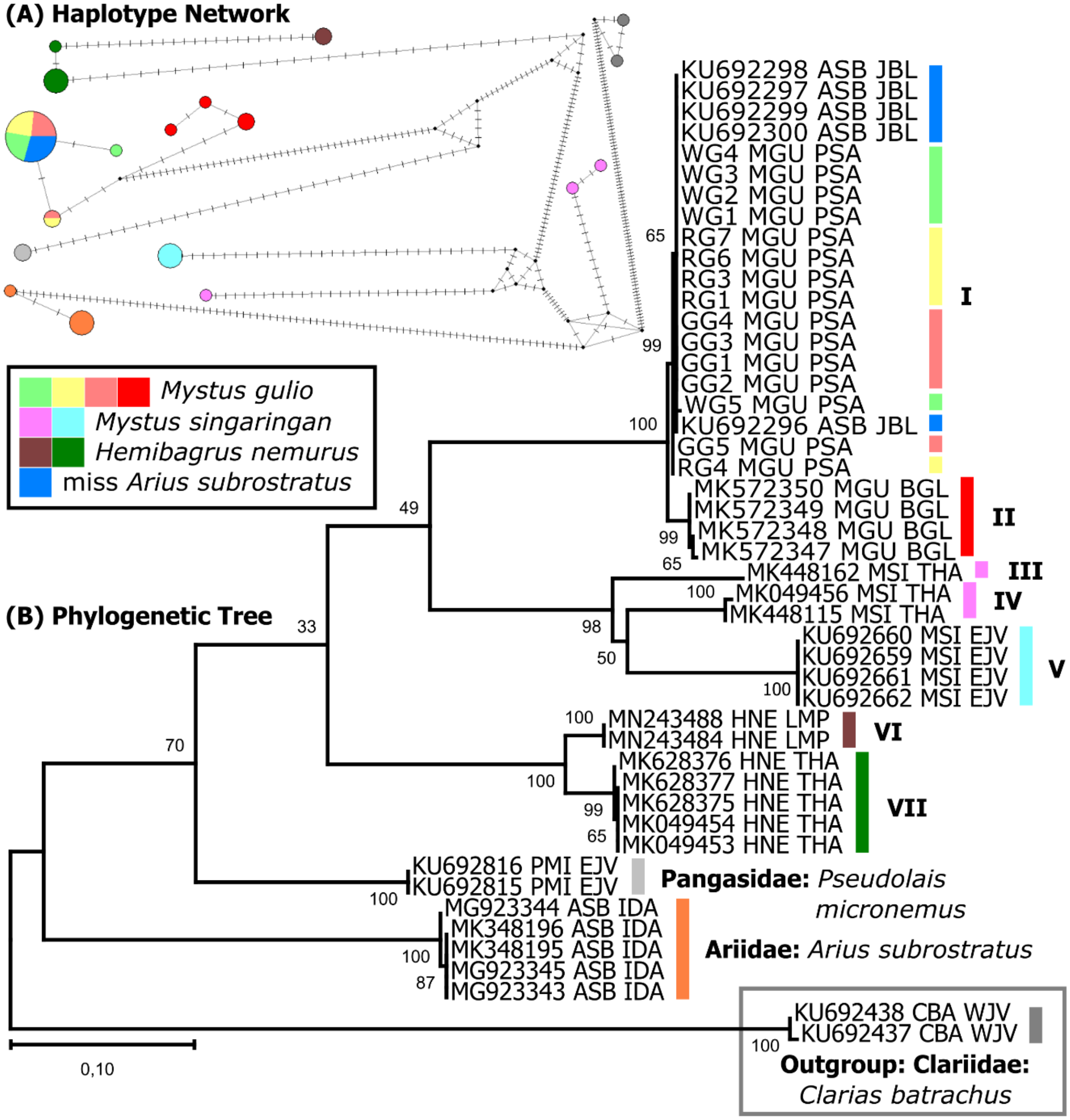

Fig. 2. Haplotype network (A) and phylogenetic tree (B) using maximum-likelihood (ML) algorithm of Hasegawa-Kishino-Yano plus gamma distributed and invariant sites (HKY+G+I) model with 591 bp sequence length. In general, the Bagridae family in the data set is separated into seven clades.

The phylogenetic analysis of the Bagridae family resulted in seven distinct clades based on the dataset used in this study. Clades I and II represent *M. gulio* from two major populations. Clade I includes the main specimen from Pasuruan, representing the Indonesian population, while clade II comprises the Bangladeshi population. The sequence of *A. subrostratus* from Cilacap, East Java, clustered within clade I, a placement further supported by the haplotype network results, indicating a high probability of misidentification. This misidentification is reinforced by the separation of the Cilacap *A. subrostratus* sequences from the Indian population, whose dataset includes accession numbers MK348195, MK348196, MG923343, MG923344, and MG923345.

The Indian population of *A. subrostratus*, representing the family Ariidae in the dataset, was clearly separated from the Bagridae family. This separation strongly supports the conclusion that the sequences from Cilacap, included in clade I, actually represent misidentified *M. gulio*, corresponding to accession numbers KU692296, KU692297, KU692298, KU692299, and KU69300.

The comparison of *A. subrostratus* sequences from Indonesia (East Indoburman) and India (West Indoburman) suggests that the true distribution of *A. subrostratus* is likely restricted to the West Indoburman region. In contrast, the separation between the Indonesian and Bangladeshi populations of *M. gulio* (clades I and II) was evident, yet not strongly significant. Both results—phylogenetic and haplotype network—showed only an 8 bp difference between the two populations, which is minimal compared to interspecific distances within *Mystus*.

This 8 bp difference is also much smaller than the intraspecific distances observed in *M. singaringan* between the Thailand populations (clades III and IV) and the East Java population (clade V), which differ by at least 60 bp. A similar pattern is seen in *H. nemurus*, where the Lampung population (clade VI) and the Thailand population (clade VII) differ by at least 24 bp. The monophyletic affinities of clades I and II suggest low speciation potential in *M. gulio*, with the possibility that the Indonesian and Bangladeshi populations are not fully geographically isolated.

Table 1. Intraclad and interclad genetic *P*-distance values. Clad groupings correspond to previous phylogenetic tree results

	a	b	c	d	e	f	g	h	I	j
a. I	**0.000**									
b. II	**0.017**	0.002								
c. III	0.184	0.176	0.001							
d. IV	0.171	**0.163**	0.105	**0.000**						
e. V	0.196	**0.203**	**0.122**	0.107	**0.003**					
f. VI	0.189	0.194	0.186	0.174	**0.209**	n/c				
g. VII	0.195	0.197	0.189	**0.169**	0.201	0.044	**0.000**			
h. Pangasidae	0.191	0.200	0.213	0.217	**0.227**	0.184	**0.176**	0.001		
i. Ariidae	0.254	0.256	0.232	0.238	**0.270**	0.261	0.269	**0.225**	**0.000**	
j. *Outgroup*	0.310	**0.333**	0.263	**0.259**	0.287	0.283	0.270	0.282	0.294	**0.003**

Light grey columns for intraclad values; dark grey columns for intraspecies values; green columns for intragenous values; pink columns for intrafamily values; light blue columns for Pangasidae interfamily values; dark blue columns for Ariidae interfamily values; orange columns for Clariidae (outgroup) interfamily values; black bold format values for lowest values and red bold format for highest values according to column category.

Genetic distance analysis using p-distance revealed several potential taxonomic issues based on the partial COI dataset used in this study (Table 1). Intraspecific distances ranged from 1.7% to 12.2%, with the lowest value observed between the Indonesian and Bangladeshi populations of *M. gulio*. These results reinforce the interpretations drawn from the phylogenetic reconstruction and haplotype network analyses, supporting the close genetic relationship between the two populations.

In contrast, intrageneric distances between *M. gulio* and *M. singaringan* were substantially higher, ranging from 16.3 to 20.3%, clearly indicating interspecific divergence within the genus *Mystus*. The intrageneric distance range within *Mystus* overlapped with the intergeneric distance range within Bagridae (16.9–20.9%), represented here by comparisons between *Mystus* and *Hemibagrus*. This overlap suggests potential taxonomic ambiguities that may warrant future revision.

Similarly, intrageneric distances within the Bagridae overlapped with interfamily distances between Bagridae and Pangasidae (17.6–22.7%). However, Bagridae remained clearly distinct from the Ariidae family, with interfamily distances ranging from 22.5 to 27%. The relatively close affinity between members of Bagridae and Pangasidae adds complexity to the interpretation of evolutionary relationships within Siluriformes.

Conversely, the pronounced genetic distance between Bagridae and Ariidae supports the phylogenetic results obtained in this study. The Bagridae–Ariidae interfamily distances also overlapped with the Bagridae–Clariidae distances (25.9–33.3%), indicating that Ariidae, similar to Clariidae used here, is appropriate as an outgroup in phylogenetic analyses of the Bagridae family.

Table 2. Results of pairwise Fst analysis of *M. gulio* populations in the data set

	1	2	3	4	5
1. Gembong River	0.000				
2. Rejoso River	–0,250	0.000			
3. Welang River	0.000	0.000	0.000		
4. NCBI: Bangladesh	0.923	0.923	0.927	0.000	
5. NCBI: Cilacap*	–0.000	–0.000	–0.000	0.948	0.000

The “*” indicates groups with previous misidentification as *A. subrostratus*. High Fst values were obtained in the comparison of each Indonesian population with the Bangladeshi population, while the Indonesian interpopulation had low Fst values.

Table 3. Results of intraspecies genetic variation analysis of *M. gulio* using nucleotide diversity (Pi) and haplotype diversity (Hd)

	Gembong River	Rejoso River	Welang River	NCBI: Bangladesh	NCBI: Cilacap
Jumlah Sequence (n)	5	5	5	4	5
Jumlah Haplotype (H)	2	2	2	3	1

Keragaman Nukleotida (Pi)	0,00068	0,00068	0,00068	0,00197	0,00000
Keragaman Haplotype (Hd)	0,400	0,400	0,400	0,833	0,000

All populations have low Pi values, and almost all populations have low Hd values, except for the Bangladesh population.

Intraspecies analyses of *M. gulio* for gene flow and genetic diversity, based on partial COI in the dataset, indicate potential and threat to *M. gulio* populations in the wild. The higher the Fst value, the smaller the potential for gene flow between the two groups **(Hutchison & Templeton, 1999)**, while values below 0.000 can be considered equal to 0.000 (Table 2). High Fst values were found in all Indonesian population groups, compared to the Bangladeshi population, indicating that these two populations are less likely to share genes. All *M. gulio* populations in Indonesia, including the population previously claimed as *A. subrostratus* in Cilacap, had Fst values of 0.000 or below, indicating that they are likely still connected and have shared genes. The proximity of all Indonesian populations indicates the possibility that *M. gulio* from both Pasuruan and Cilacap are still a single population, so the potential distribution pattern of *M. gulio* is extremely broad, and not blocked by geographical boundaries, including ecoregion boundaries. The potential for wide population distribution of *M. gulio* in Indonesia is associated with low genetic diversity based on nucleotide diversity (Pi) and haplotype diversity (Hd) values (Table 3). Hd values are generally considered high if ≥0.500 and Pi values are generally considered high if ≥0.005 **(Lamadi *et al.*, 2023)**. All populations had low Pi values ranging from 0.00000 to 0.00197, and almost all populations had low Hd values, ranging from 0.000 to 0.400, except for the Bangladesh population with an Hd value of 0.833. The low population diversity, coupled with the wide distribution of *M. gulio* populations in Indonesia, indicates the vulnerability of these populations to environmental threats, as well as the limited broodstock for interpopulation breeding that can rapidly increase genetic diversity, especially populations in Java.

Molecular misidentification based on BLAST results often occurs due to the similarity of morphological characteristics, leading to misidentification or taxonomic conundrums. Genetic misidentification, due to limited systematic identification literature, occurs in some members of the order Gobiiformes, such as the genus *Giuris*. Almost all sequences in the gene bank belong to *Giuris margaritaceus*, but when tested with phylogenetics, haplotype networks and genetic distances, the sequences are separated into several different species clades **(Lamadi *et al.*, 2023)**. Furthermore, the morphological similarity of Bagridae-Ariidae is much higher, characterized by cases of molecular misidentification, while the morphological similarity of Bagridae-Pangasidae and Ariidae-Pangasidae, tends to be low, with no cases of misidentification found. Similarities in morphological, physiological and behavioral adaptations are often not directly proportional to genetic information, because these adaptations can be caused by analogous processes, not homologous mechanisms. Likewise, the orders Scombriformes and Istiophoriformes, which were originally both groups, converged on the order Scombriformes. The morphological, behavioural and physiological similarities of the Xiphiid members (Xiphiidae and Istiophoridae), with the Thunnini tribe in the family Scombridae, led to their initial thought to be derived from a common ancestor **(Johnson, 1986; Collete *et al.*, 2001)**, but phylogenetic results later refuted this hypothesis **(Orrell *et al.*, 2006; Betancur *et al.*, 2013)**. The potential for misidentification and misclassification due to similarities in morphology, physiology and behavior is quite common, as is the case of the misidentification of *A. subrostratus* from Indonesia in this study dataset, as well as the Bagridae family intergenus and interfamily cases in the dataset.

Geographic isolation of freshwater species has a major impact on their distribution, with some taxa potentially subject to misidentification due to overly broad distributions. The distribution of *M. gulio* has been considered suspicious, as the distribution range of the species from West Indoburman to East Indoburman is unusual in the Bagridae family. Commonly, genetic distances based on partial COI ≥2 % in Teleostei fishes, can increase the potential for the two sequences belonging to separate species **(Bañón *et al.*, 2022)**, so the suspicion of *M. gulio* of West and East Indoburman populations in this dataset is reduced. Some people use the name *M.* aff. *gulio* or *Mystus abbreviatus* to address specimens from East Indoburman, especially from Java, and with the findings of this study, there is potential for *M. abbreviatus* to become a synonym of *M. gulio*. In general, *M. gulio* has a wide distribution, from South Asia such as Bangladesh, Nepal, Pakistan, India, Sri Lanka, to Southeast Asia such as Vietnam, Thailand, Malaysia, and Indonesia **(Gupta, 2014)**. Various taxa that cannot migrate by sea are separated by geographical barriers around Myanmar, so the biodiversity of West and East Indoburman is extremely different, although some have high morphological similarity due to homologous factors. One such case of misidentification based on geographic isolation is *Notopterus notopterus*, which is morphologically very similar, but after phylogenetic analysis, haplotype networks and genetic distances, the two populations were declared as two different species. The population in East Indoburman was then suggested to be called *Notopterus sylurus*, with a genetic distance of 7 to 8.8% **(Lavoué *et al.*, 2020)**. This was also the case for *Channa gachua*, with a genetic distance of 8.1% **(Conte-Grand *et al.*, 2017)**. Both *M. gulio* populations may still be sharing genes, although not intensely, or at least have expanded their distribution by sea, during the recent divergence. The unusual distribution of *M. gulio* can also be suspected from the similarity of the Pasuruan population to Cilacap, thus studies of *M. gulio* biology will be helpful in the future.

Low genetic diversity reflects poor population health, but genetic similarity between populations that cross geographical boundaries increases the potential ability of fish to migrate by sea and not be restricted to a single area. Populations with limited ranges face a greater threat of extinction, especially in freshwater endemic species **(Chua**

***et al.*, 2019)**. Freshwater and brackish species with low range capabilities generally have their gene flow restricted by geographical and ecological boundaries such as ecoregions, so the distribution of species in more than one ecoregion expands the possibility of obtaining broodstock with different genetic information to increase genetic diversity, as in the case of the genus *Oryzias* in the Sundaland. The discovery of *Oryzias hubbsi* in different ecoregions, confirmed through morphology and partial COI genetics, can serve as the basis for its preservation through *ex situ* conservation **(Paricahya *et al.*, 2024a, b)**. The Pasuruan and Cilacap populations of *M. gulio* are separated by different ecoregions. The Pasuruan area belongs to the Central and East Java (CEJ) ecoregion, while Cilacap belongs to the Indian Slope of Sumatra and Java (ISJ) ecoregion **(Abell *et al.*, 2008; Chua *et al.*, 2019)**. Similarity of genetic information in distant populations may be an obstacle to *in situ* conservation of *M. gulio* species, such as for aquaculture in the future. Parental diversity can help increase heredity variation, in search of superior breeding stock for domestication. Low genetic diversity can be caused by factors such as habitat destruction, inbreeding and blockage of migration pathways **(Phadphon *et al.*, 2019)**. Several studies have been conducted on *M. gulio* as related to growth performance based on sex **(Bagum *et al.*, 2009)**, morphology, reproduction, feeding, growth patterns **(Gupta, 2014)**, aquaculture, and captive breeding **(Kumar *et al.*, 2019)**; however, this species has not been commonly utilized as an aquaculture commodity in Indonesia.

CONCLUSION

The COI-based analysis used in this study to confirm *M. gulio* populations from the Rejoso, Welang, and Gembong Rivers in Pasuruan, East Java, revealed several important conclusions. Molecular identification confirmed all three populations of specimens in this study to be *M. gulio*, although there was a misidentification on the NCBI website for *M. gulio* in Cilacap. The genetic diversity between riverine populations in Pasuruan is very low, and even has high similarity with the Cilacap population, indicated by low pairwise Fst values. Low genetic diversity can increase the potential for local decline and extinction of these populations. The relationship between the Pasuruan population and the Cilacap population indicates that there is potential for unusual dispersal of Bagridae by the ocean, specifically in *M. gulio*. The hypothesis of marine dispersal (with particular note) can also be considered through the wide distribution of *M. gulio*, ranging from Western Indoburman such as Bangladesh, to Eastern Indoburman such as Indonesia. The order Silluriformes has some overlap between members through genetic p-distance analysis, and this could be a taxonomic conundrum to consider in future research. Domestication and other conservation efforts such as area management, can be conducted considering the genetic vulnerability of *M. gulio* in Pasuruan, even in Java.

ACKNOWLEDGEMENTS

The authors would like to thank the Universitas Brawijaya for financially supporting this research, through the Professor Research Grant and the various parties who assisted during the research process.

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