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RESEARCH PAPER

First Identification DNA Barcoding of Bronze Featherback Fish, *Notopterus notopterus* (Pallas, 1769) (Osteoglossiformes: Notopteridae), in Brantas River, East Java, Indonesia

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Abstract

Bronze featherback fish *Notopterus notopterus* is a family of notopteridae native to river drainage in South and Southeast Asia, which are distributed in India, Pakistan, Bangladesh, Malaysia, Myanmar, Laos, Cambodia, Vietnam, Thailand, and Indonesia. This study aims to identify the molecular phylogenetic of *Notopterus notopterus* in Java, Indonesia based on the Cytochrome Oxidase subunit I (COI) gene. This specimen from the brantas river has 680 base pairs with 99.84% identity value for *Notopterus notopterus*. This is the first time molecular phylogenetic reported of bronze featherback fish in Brantas River, Indonesia

Introduction

The bronze featherback fish, *Notopterus notopterus* (Family Notopteridae), is a popular food fish with ornamental value and thrives well in freshwater rivers, ponds and lakes. Members of a group commonly called “knife fish” are genus *Notopterus, Chitala, Papyrocranus* and *Xenomystus*, which are distributed widely in Africa, the South and Southeast Asia (Borkhanuddin et al., 2020). Currently, the only valid species of the genus *Notopterus*, is one of such widely-distributed species, occurring from the Indus basin (Pakistan, India and Bangladesh) in the west, to the Mekong region – slightly extending east to the Annamite Range - (Cambodia, Malaysia, Myanmar, Laos, Thailand and Vietnam) in the east and to Sumatra and Java (Indonesia) in the south (Memon et al., 2021; Lavoué et al., 2020). In Indonesia, this species is found in Java, Sumatra, and Borneo (Rapita et al., 2021).

The bronze featherback fish (*N. notopterus*, Pallas 1769) is one of Indonesia's endemic fish (Sukendi et al., 2020). In Java, *N. notopterus* is quite widespread in fresh waters almost all over the mainland which includes Central Java and East Java. However, currently, the existence of this species continues to decline drastically (Yulindra et al., 2017). Even the results of the interview of Rapita et al. (2021) with local fishermen in Sei Gesek Reservoir, Bintan Regency, Riau (Indonesia) found the fact that the catch of bronze featherback fish was not much and sometimes even not found. The sharp decline

in the number was allegedly caused by the high selling price of fish (IDR 15,000 – IDR 30,000 per living fish), resulting in intensive fishing (Rapita et al., 2021), without being balanced with adequate conservation and cultivation efforts. In addition, in their research, Wibowo & Sunarno (2006) also stated that bronze featherback fish have a relatively small fecundity (442-11,972 eggs). The government through the Decree of the Minister of Maritime Affairs and Fisheries in 2021 has also stated that the bronze featherback fish (*N. notopterus*, Pallas 1769) is one of the protected fish species because it is threatened with extinction. As previously mentioned that bronze featherback fish spread to the east of Java island, and the Brantas River, Mojokerto Regency is one of their habitats (Nugroho et al., 2020).

The existence of *N. notopterus* in Brantas River, East Java has been recorded (Nugroho et al., 2020), but the molecular identification especially the DNA barcode of *N. notopterus* has not been studied specifically. In Indonesia, the DNA barcode of *N. notopterus* has only been reported on NCBI GenBank from Sumatra (South Sumatra: Musi Banyuasin Regency) (Wibowo & Sunarno, 2006; Lavoué et al., 2020) and Java (Central Java: Rawa Pening Lake) (Dahruddin et al., 2016; Lavoué et al., 2020). DNA barcodes are needed to determine kinship or lineage relationships at the molecular level of a type of fish, which later these molecular markers can be used to support the interests of conservation and cultivation of a type of fish (Ghouri et al., 2020), the context of the fish in this study is *N. notopterus*.

The study used the DNA Barcoding method with a specific gene in the mitochondrial genome (mtDNA), the Cytochrome C Oxidase subunit I (COI) gene (Hebert et al., 2003; Liu et al., 2014). DNA barcodes based on the partial nucleotide sequence of the mitochondrial gene Cytochrome C Oxidase I (COI) can serve as the core of a global bio-identification system for animals (Hebert et al., 2003). DNA barcoding for the identification of freshwater fish diversity in Indonesia has been widely used (Pramono et al. 2017, Saleky & Dailamy, 2021), Asian Redtail Catfish (Syaifudin, 2017), Snakeskin Gourami and Blue Gourami (Syaifudin, 2019), Striped Snakehead and Ocellated Snakehead fish (Syaifudin, 2020). The aim of this study is molecular identification record the identification DNA barcode of *N. notopterus* in Brantas River, East Java, Indonesia.

Materials and Methods

Sample Collection

A total of 10 samples were collected on September 7th, 2021 from the Brantas River, Mojokerto Regency, East Java (7°27'44.1"S 112°25'43.6"E) (Figure 1). The tissue samples were obtained from the anal fin of bronze featherback fish (Figure 2). First of all, the surface of the fish sample was cleaned by aquadest and

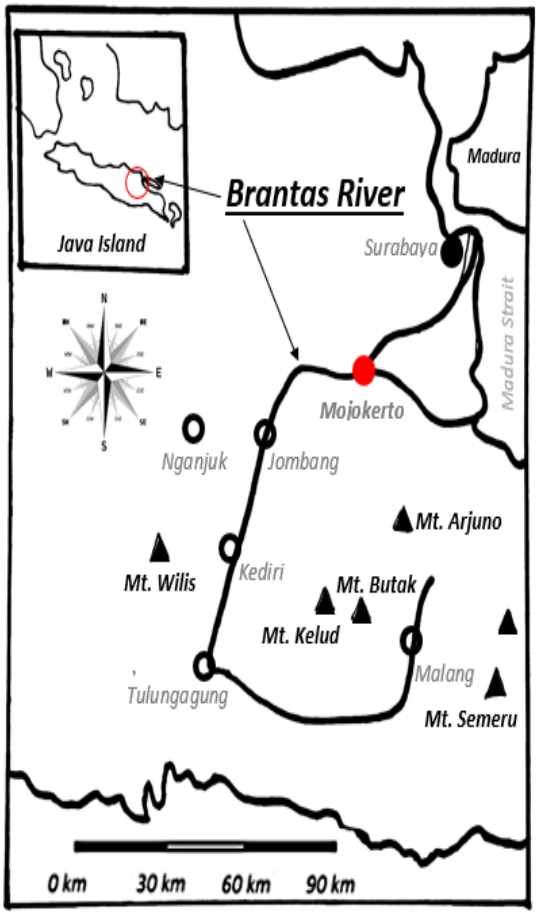

Figure 1. Geographical location of the sampling stations: red point.

then insert into a 2 mL Cryotube which already contains 96% ethanol liquid followed by writing the sampling code "21PPR_JMB_01" and so on. After that, the sample was ready to proceed to the next stage i.e DNA Extraction stage.

Sixteen (16) sample sequences COI gene were downloaded from NCBI GenBank, thirteen (13) *N. notopterus* (KM213053.1; KU692675.1; MK049497.1; AP008925.1; MW343514.1; KT022089.1; MK448091.1; MT328861.1; MK628319.1; JX901490.1; LC189928.1; MK572369.1; MT434341.1), one (1) *Chitala lopis* (KM213054.1), one (1) *Xenomystus nigri* (MK074697.1), and one (1) *Papyrocranus afer* (JF510515.1).

Molecular Detection

DNA extraction was performed using Geneaid Gsync kit extraction by following the manufacturer's procedure. The 2% gel electrophoresis was used to visualize and check the DNA extraction result. PCR methods using a primer Fish F1 (5'TCA ACC AAC CAC AAA GAC ATT GGC AC-3') and Fish R1 (5'-TAG ACT TCT GGG TGG CCA AAG AAT CA3') (Ward *et al*., 2005).

The process of PCR amplification was carried out in 40 cycles. The predenaturation stage was carried out at a temperature of 94°C for 2 minutes, denaturation at 94°C for 45 seconds, annealing at a temperature of 45°C for 45 seconds, extension at 72°C for 1.5 minutes, and final extension at 72°C for 10 minutes. This cycle is repeated 40 times using Biosystems™ Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific). After the DNA amplification process, 2% gel electrophoresis was used to visualize and check the DNA extraction result. Electrophoresis was carried out by taking 4 µl of amplified DNA which was inserted into an agarose well, dissolved in the TAE buffer then stained using GelRed™ and could be electrophoresed at a voltage of 110 V for 20 minutes. The mtDNA sequence analysis was performed on sequencing machine, ABI 3500 Genetic Analyzer (Thermo Fisher Scientific).

Figure 2. Specimens of *Notopterus notopterus* was captured on 7 September 2021 in the Brantas River, Mojokerto Regency, East Java.

Table 1. COI Primers used for PCR amplification.

Primer	Sequence (5' → 3')	mers	Target group	Reference
FishF1 (forward)	TCAACCAACCACAAAGACATTGGCAC	26	Fish	Ward *et al*., 2005
FishR1 (reverse)	TAGACTTCTGGGTGGCCAAAGAATCA	26	Fish	Ward *et al*., 2005

Table 2. Sequencing result of *N. notopterus* from the Brantas River, Mojokerto Regency, East Java.

DNA Barcoding
CNN ACT CGT ATT TGG GGC TGA GCA GGC ATA GTA GGC ACA GCC CTA AGC CTG CTG ATC CGA GCA GAA TTG AGC CAA CCC GGC TCA CTG CTT GGC GAC GAC CAA ATC TAT AAT GTT ATC GTA ACA GCA CAC GCC TTC GTA ATA ATT TTC TTT ATG GTA ATG CCT ATC ATG ATT GGA GGC TTC GGA AAT TGA CTA ATT CCC CTA ATA ATT GGA GCC CCT GAT ATA GCC TTC CCA CGA ATA AAT AAT ATA AGC TTC TGA CTT CTA CCC CCA TCC TTC CTA CTG CTC CTA GCC TCT TCA GGA GTA GAA GCC GGT GCC GGA ACA GGA TGA ACC GTA TAT CCG CCT TTA GCA GGA AAC CTA GCA CAT GCA GGC GCC TCC GTT GAT CTT ACA ATT TTT TCA CTT CAT CTT GCC GGT GTG TCC TCA ATT CTA GGG GCC ATT AAT TTT ATT ACA ACT GTA TTT AAT ATA AAA CCA CCC GTA GTT TCA CAA TAC CAA ACA CCA CTA TTC ATC TGA GCT GTA ATA ATT ACT GCA GTT TTA CTC CTT TTA TCA CTT CCG GTC TTA GCC GCC GGC ATT ACA ATG CTT CTC ACA GAC CGC AAC CTC AAC ACA ACA TTC TTC GAT CCG GCA GGC GGG GGC GAT CCG ATC CTT TAT CAA CAC TTA TTT TGA TTC TTT GGC CAC CAA AAA NNN TCT NN

This research was conducted by analysis the sequence using the NCBI BLAST (Basic Local Alignment Search Tools), where the sequence was obtained from NCBI GenBank. The selected Cytochrome Oxidase I (COI) sequences will be stored using the FASTA format for further analyzing using the NCBI BLAST. The sequence alignment was carried out by editing/alignment builds via ClustalW in MEGA X program (Kumar et al., 2018). The phylogenetic tree was created based on the Neighbor-Joining (NJ) analysis tree method in the MEGA X program (Kumar et al., 2018) based on Kimura-2-Parameter (K2P) model (Kimura, 1980). The Evolutionary analyses were conducted in MEGA X program (Kumar et al., 2018) based on Kimura-2-Parameter (K2P) model (Kimura, 1980), the number of base substitutions per site from between sequences are shown.

Results

In this study, the DNA Barcoding of the specimen from the Brantas River (Mojokerto Regency, East Java) were successfully sequenced with a base-pair length of 615 bp by using the Fish F1 and Fish R1 primers (Ward *et al*., 2005). The sequence data from this research sample (21PPR_JMB) has also been submitted to GenBank NCBI with Accession Number OP872712.

The specimen from the Brantas River was compared to the NCBI GenBank (National Center for Biotechnology Information) and identified as *N. notopterus* with the most identical to *N. notopterus* from South Sumatra and Central Java (Rawa Pening Lake) because our samples have 100% Percent Identity value against both, then Query Coverage value of 99% for Central Java and 100% for South Sumatra, and both of them have E-value of 0.0 (Table 1). The results of this study indicated that the Cytochrome C oxidase subunit I (COI) gene had a high level of accuracy for species identification, especially for fish identification.

This study not only presents a comparison of Query Coverage and Percent Identity value between our samples of *N. notopterus* with species of *N. notopterus* from various locations but also with other species belonging to the same Family: Notopteridae (i.e. *Chitala lopis* from Sumatra, Indonesia; *Xenomystus nigri* from the Republic of the Congo; *Papyrocranus afer* from Nigeria) based on BLAST analysis. The result is that the samples of this study on *Chitala lopis* from Sumatra, Indonesia (KM213054.1) have the same 100% of Query Coverage and 88.7% of Percent Identity value; on *Xenomystus nigri* from the Republic of the Congo (MK074697.1) have the same 99% of Query Coverage value and 81.09% of Percent Identity value; on *Papyrocranus afer* from Nigeria (JF510515.1) have the same 98% of Query Coverage value and 80.72% of Percent Identity value (Table 1). The estimates of evolutionary divergence of our samples on *Chitala lopis* from Sumatra, Indonesia (KM213054.1) have 0.12940 value; on *Xenomystus nigri* from the Republic of the Congo (MK074697.1) have 0.22270 value; on *Papyrocranus afer* from Nigeria (JF510515.1) have 0.23638 value (Table 2)**.**

Discussion

Phylogenetic tree results from the Neighbor-Joining method (Saitou & Nei, 1987) showed that the samples from Brantas River, Mojokerto Regency, East Java are in one subclade (SC I) with *N. notopterus* from South Sumatra (KM213053.1) and Central Java: Rawa Pening Lake (KU692675.1) (Figure 3). On the phylogenetic tree, it can also be seen that *N. notopterus* originating from South Asia (i.e. India, Bangladesh, Pakistan) has its clade (C II). Previous studies already stated that *N. notopterus* was found in India, Bangladesh, and Pakistan (Abbas et al., 2013). *N. notopterus* originating from Southeast Asia (i.e Indonesia, Malaysia, Myanmar, Thailand) is in another

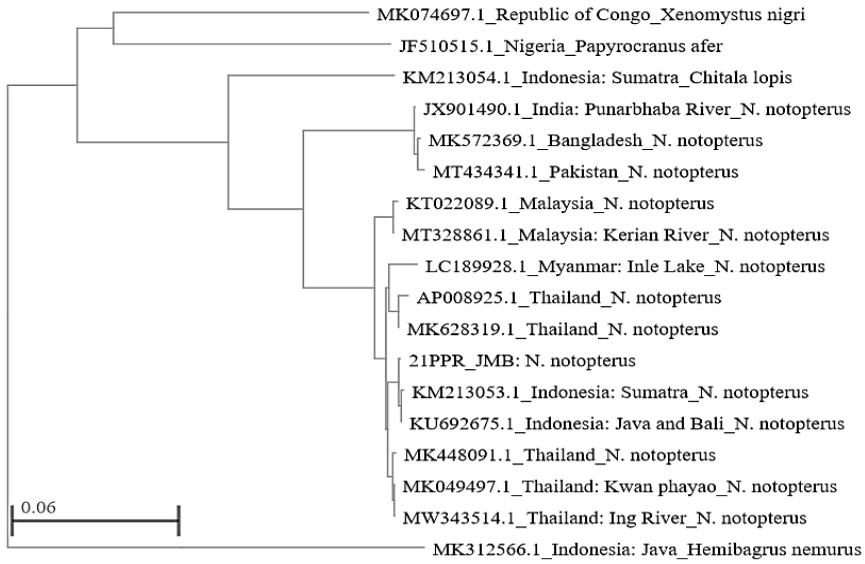

Figure 3. Phylogenetic tree of *N. notopterus* based on COI Gene.

clade (C I), and within that clade forms the same subclade based on the region of the country with N. notopterus (KT022089.1 and MT328861) located in one subclade (SC V), both taken from waters of Malaysia. *N. notopterus* (LC189928.1) from Myanmar waters located at (SC III) while *N. notopterus* (AP008925.1 and MK628319.1) from Thailand waters located at (SC IV). SC IV is a branch of SC III where the territory of Thailand and Myanmar, when viewed from a geographical location, is known to have some areas that directly border. This causes the three samples from the two countries to have closer lineage in Neighbor-Joining tree data. In addition, *N. notopterus* (MK049497.1, MK448091.1, and MW343514.1) which also comes from Thailand waters is in a separate subclade (SC II). It indicates that the three samples do not come from the lineage of *N. notopterus* which inhabits Myanmar waters directly like other *N. notopterus* originating from Thailand waters in SC IV. The estimates of evolutionary divergence of our samples *N. notopterus* (21PPR_JMB) on *N. notopterus* from South Sumatra (KM213053.1) have 0.00000 value and Central Java: Rawa Pening Lake (KU692675.1) have 0.0000 value (Table 2). The value (0.00000) in estimates of evolutionary divergence indicates that the two samples have the same genetic makeup (identical). This is presumably because the *N. notopterus* sample (21PPR_JMB) came from the original *N. notopterus* parent fish from Indonesia as well as *N. notopterus* (South Sumatra: KM213053.1 and Central Java: KU692675.1). In addition, it may also be due to the relatively similar aquatic environmental conditions throughout Indonesia. According to previous research, the environment affects genetic diversity in fish (Van Leeuwen et al., 2018).

Our samples were compared to the GenBank NCBI (National Center for Biotechnology Information) and identified as *N. notopterus* with the most identical to *N. notopterus* from South Sumatra and Central Java (Rawa Pening Lake). It is because our samples have 100% Percent Identity value against both, then a Query Coverage value of 99% for Central Java and 100% for South Sumatra, and both of them have an E-value of 0.0 (Table 1). According to Hebert et al. (2003), species with 99-100% similarity levels are identical. *N. notopterus* from Thailand (MK049497.1; MW343514.1; MK448091.1; MK628319.1) can still be said to be identical to our sample as well because they have a Percent Identity value above 99% with our samples. Regarding the Query Coverage value which is 99% of *N. notopterus* from Central Java: Rawa Pening Lake. This result is caused by mutations at the DNA level (deletions or insertions) caused by inbreeding (Wang et al., 2001) and the homogenizing effect of gene flows (Doellman et al., 2018). In Rawa Pening Lake (Central Java) considering that the Lake is a closed ecosystem, it has a higher chance of inbreeding. Not with river and sea ecosystems which are open ecosystems. The phylogenetic tree result from the Neighbor-Joining method showed that our samples from Brantas River (Mojokerto Regency, East Java) are in one subclade (SC I) with *N. notopterus* from South Sumatra and *N. notopterus* from Central Java (Figure 3). This proves that our *N. notopterus* samples are more closely related to the two than the others.

This DNA barcode information of *N. notopterus* from the Brantas River (East Java) can be used as reference data and becomes essential for genetic resources conservation efforts and the cultivation of fish considering that this fish is threatened with extinction in Indonesia, including in the Brantas River, East Java (Khairul et al., 2020; Yulindra et al., 2017). This research will also be useful for researchers around the world who

Table 3. Species Identification and Similarity

Species	Location	Accession ID	Query Coverage (%)	Percent Identity (%)
			21PPR_JMB (*Notopterus notopterus* from Brantas River)	
Notopterus notopterus	Indonesia: Sumatra	KM213053.1	97	99,55
Notopterus notopterus	Indonesia: Java and Bali	KU692675.1	94	99,84
Notopterus notopterus	Thailand: Kwan Phayao	MK049497.1	95	99,38
Notopterus notopterus	Thailand	AP008925.1	97	98,49
Notopterus notopterus	Thailand: Ing River	MW343514.1	93	99,37
Notopterus notopterus	Malaysia	KT022089.1	97	98,03
Notopterus notopterus	Thailand	MK448091.1	92	99,21
Notopterus notopterus	Malaysia: Kerian River	MT328861.1	95	98,30
Notopterus notopterus	Thailand	MK628319.1	92	99,05
Notopterus notopterus	India: Punarbhaba River, Malda, West Bengal	JX901490.1	95	92,74
Notopterus notopterus	Myanmar: Shan State, Nyaung Shwe, Inle Lake	LC189928.1	93	98,28
Notopterus notopterus	Bangladesh: Chittagong Division, Rangamati, fish landing pier	MK572369.1	95	92,58
Notopterus notopterus	Pakistan	MT434341.1	90	92,54
Chitala lopis	Indonesia: Sumatra	KM213054.1	97	88,40
Xenomystus nigri	Republic of the Congo	MK074697.1	94	81,00
Papyrocranus afer	Nigeria	JF510515.1	93	80,56
Hemibagrus nemurus	Indonesia: Java	MK312566.1	68	75,91

will focus on the genetics and lineage of this species (Nugroho et al., 2020). Considering the DNA barcode in this study has been able to explain the genetic relationship of bronze featherback fish (*N. notopterus*) originating from the Brantas River, Mojokerto Regency, East Java to the same species originating from various locations. This information plays a crucial role in conserving genetic resources and cultivating bronze featherback fish. Using this DNA barcode, we will be able to breed individuals with higher survival rates by implementing selective breeding (high genetic diversity) to suppress inbreeding (low genetic diversity) in a population. Selective breeding activity is the most important aspect of efforts to conserve the genetic resources of a fish species because in practice since it can determine which individuals will be selected as brooders for the next better generation (Hadie et al., 2014). Frankham (2003) has stated that individuals with high genetic diversity (non-inbreeding) tend to have a greater chance of survival because they are considered better able to adapt to climate change, new diseases or pests, pollution to habitat destruction. Therefore, it is crucial to conduct this research to support efforts to conserve genetic resources and build a superior bronze featherback fish farming ecosystem to prevent the extinction of the *N. notopterus* species which is becoming increasingly evident in Indonesia, especially Brantas River.

Conclusion

In conclusion, we found that our samples from the Brantas River have 615 base pairs with 100% Percent Identity value with *N. notopterus* obtained from South Sumatra (KM213053.1) and Central Java: Rawa Pening Lake (KU692675.1) sample of GenBank NCBI. This is the first identification DNA barcode of bronze featherback fish *N. notopterus* in Brantas River, Mojokerto Regency, East Java, Indonesia. Our sample (21PPR_JMB) has also been submitted to GenBank NCBI with Accession Number OP872712.

Ethical Statement

Specific permission was not required to conduct sampling for this research. No experiments have been carried out using living organisms. The authors confirm that the field studies did not involve any endangered or protected species.

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Author Contribution

The research was design by Wulansari and Jamal. Laboratory and molecular analysis were done by Khansa, Farizky, Sani, and Madduppa. Field studies was carried out by Khansa and Farizky. All authors contributed to the writing of the article.

Conflict of Interest

The authors declare that they have no conflict of interest that can influence the work reported in this paper.

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