Phylogenetic of Featherback Chitala sp from South Sumatra Based on Cytochrome C Oxidase Subunit I (COI) Gene

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Phylogenetic of Featherback Chitala sp from South Sumatra Based on Cytochrome C Oxidase Subunit I (COI) Gene

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Abstract. The featherback *Chitala* sp in Indonesia inhabit riverine of Sumatra, Java and Kalimantan. Featherbacks have been protected as threatened species. This research aims to identify the sequence of Cytochrome C *Oxidase* Subunit I (COI) gene of mtDNA, and construct the phylogenetic trees between species of featherbacks in South Sumatra. This research was conducted in March–August 2020. The methods used in barcoding species and determining phylogenetic i.e., DNA isolation, DNA amplification using PCR and sequencing of COI gene regions of mtDNA. The COI gene was sequenced by PCR (*Polymerase Chain Reaction*) with optimum annealing temperature 50^0C for 30s with 35 cycles. After editting, sequence length of the COI gene of featherbacks was 621 base pairs (bp) nucleotide. Based on BLASTanalysis, specimen CLP2 from PT PLN Indralaya had the highest similarity (99.28%) to *C. lopis* (Malaysia), then 98.88% to the same species from Kampar River, Riau. Speciemens from Musi River (CLS1, CLS 3) and PT PLN (CLP3) indicated the highest similarity of 95.19% with *C. chitala* from India. The phylogenetic trees showed that Chitala formed four sub-clusters and it was clearly distant between species *C. lopis* and *C. chitala* (bootstrap value =73).

1. Introduction

Chitala is a genus of fish of the family Notopteridae. Based on Fishbase (2019), there are 6 species of *Chitala*, namely *Chitala lopis*, *C. blanci*, *C. borneensis*, *C. chitala*, *C. hypselonotus* and *C. ornata*. They are native to freshwater in South and Southeast Asia, and commonly known as the Asian knifefishes or featherbacks. Three of them are found in Indonesia, namely giant featherback *Chitala lopis* (Sumatra, Java and Borneo), *C. borneensis* and *C. hypselonotus* (Borneo and Sumatra). The distribution of featherbacks in the Musi River was in the downstream to the middle zone such as the tidal area around Borang, Meriak River, to Musi Rawas [1]. *C. chitala* distributed in Asia, including Indus, Ganges-Brahmaputra and Mahanadi river basins in India, however it was reported in Malaysia and Indonesia based on *Chitala lopis* [2], while *Chitala lopis*, the largest fish in the genus, known as the giant featherback or Indonesian Featherback is a freshwater fish discovered in India, Pakistan, Bangladesh, Sri Lanka, Nepal, Thailand, and Indonesia [3].

The annual production of featherbacks in Indonesia has continued to decline steadily from 8,000 tonnes (1991), 5,000 tonnes (1995) to 3,000 tonnes (1998) [4]. The annual production of this species in the Kampar River has also decreased sharply from 156.9 tonnes (2014) to 123.9 tonnes (2015) [5]. *Chitala lopis* found in river drainages in Western and Central Java has not been recorded from this region in over 160 years therefore it has been evaluated as Extinct. Threats in the region have resulted in habitat degradation extremely that has directed to the extirpation of many large-bodied freshwater fish species, and include pollution from industrial, domestic, and agricultural inputs, extensive and unsustainable fishing activities, land conversion acutely for urban and agricultural growth [6]. With the significant decrease in biodiversity, species extinction raises the need for the conservation of

biodiversity [7]. Species identification supported by DNA barcoding will allow more information on fish diversity to the fisheries managers and ecologists who create the policies for the conservation and sustainable of fisheries resources [8].

The majority of the fish species described to date have been identified using a traditional analyses of morphological charac, thus not accounting for the existence of cryptic taxa, and many areas are still to be explo [9]. DNA barcoding is a method of fast species identification usi short DNA sequences [10]. The cytochrome oxidase subunit I (COI) gene is a protein coding in mitochondrial DNA that has been widely used as a tool for identifying animal species, investigate evolutionary process with high resolution [11] and exhibits 5-10 times higher vari ility than single copy nuclear genes [12]. Current study has successful to use DNA barcoding to facilitate decision makings and selections for biodiversity, breeding and conservation in fishery management [13].

COI gene has been widely used for barcoding DNA for fish in Australia [14], the Persian Gulf [15], tilapia [16], *Channa striata*, *C. pleuropthalma* [17], and *Chitala* sp (18). Four featherbacks of the family Notopteridae in Thailand have been characterized using allozyme (19). DNA barcode has been used to identify *Chitala lopis* in Kampar, Indragiri Hilir, Musi and Mahakam River (18), and examine the phylogeographic structure of the Asian bronze featherback *Notopterus notopterus* [20]. This research was conducted to obtain nucleotide sequences of the COI gene through DNA barcoding, which can then be used to determine the genetic relationship and conservation of featherbacks originating from the Musi River in Beruge Village, Babat Toman District, Musi Banyuasin Regency and PT PLN (Persero) Indralaya, Ogan Ilir Regency in South Sumatra.

2. Methodology

2.1. Sample collection

Four specimens and water samples were collected from 2 locations (Figure 1), the Musi River in Musi Banyuasin Regency (coded as CLS) and domesticated fish at PT PLN (a state owned company in the electricity sector) Indralaya, Ogan Ilir Regency (coded as CLP) at South Sumatra Province Indonesia. For each specimen, approximately 4 cm of a gment of the caudal fin was dissected with a sterile blade, preserved in 96% ethanol (1:10 w:v) then stored in 1.5 ml Eppendorf tubes at -20°C until further step.

2.2. DNA Extraction

A total of four fin clips from two locations have been used in genomic DNA extraction. Total genomic DNA was extracted based on Geneaid DNA Extraction kit (GT 100 Geneaid Biotech Ltd. Taiwan) as outlined in the manufacturer's guidance. An RNAse incubation procedure was added to reduce RNA contamination. DNA samples were further kept in freezer (-20ºC) until required.

2.3. DNA Amplification

Featherbacks DNA was used in PCR with primer pairs of FishF2-5'TCGACTAATCATAAAGATATCGGCAC3' and FishR2-5'ACTTCAGGGTGACCGAAGAATCAGAA3' to amplify 650 bp fragment [14]. PCR s performed in a final volume of 50 µl using MyTaq™ Red Mix (Bioline). Each reaction contained 1 of 10 µM each primer, 20 µl of nuclease-free water, 25 µl *myTaq olymerase red mix* and 3 µl of DNA template. The thermal cycling protocol was as follows: initial denaturation at 95°C for 1 min (1 cycle) followed by 35 cycles of 95°C for 15 sec, annealing at 50ªC for 30 sec, extension or elongation at 72°C for 15 and a final extension at 72°C for 4 min. Furthermore, PCR products were run in electrophoresis 1% agarose gel in 1x TAE Buffer at 75V for 35 minutes and visualized to determine the DNA bands using Gel Documentation. DNA samples that were successfully amplified using PCR were then sequenced at 1st Base DNA Sequencing Service.

2.4. Data Analysis

Four samples of sequencing from both directions were saved in Fasta format. The resultant fragments were approximately 680 - 698 base pairs (bp). After trimming process with MEGA (Molecular Evolutionary Genetics Analysis) version 7 [22], the sequences had 621 bp length and no

gaps within sequences. The sequences were checked their identity using BLAST (Basic Local Alignment Search Tool) in NCBI (National Center for Biotechnology Information). For sequence comparisons, pairwise genetic distances were quantified based on the Kimura 2-parameter (K2P) distance model [23]. The phylogenetic tree of featherbacks was constructed using the Neighbor-Joining (NJ) method. In the phylogenetic construction, *Oreochromis niloticus* (KM438538.1) [16] was also added as species outgroup.

3. Results and Discussion

3.1. The Sequence Identity

The BLASTn analysis showed that Featherbacks COI sequences in this study concordance with those in the GenBank database. Sample (CLP2) has different nucleotide sequences from fish samples (CLP3, CLS1, and CLS 3). CLP2 had the highest percentage of identity (99.28%) to *Chitala lopis* from Malaysia (Accession code KT001050.1) and 98.88% to the same species from Kampar River, Riau (Indonesia) (KM213054.1), but showed lower percentage of identity (92.49-94.55%) to *Chitala chitala* from India and Bangladesh (Accession code FJ459465.1 and MF140393.1). Samples CLP3, CLS1 and CLS3 had 95.19% identity to Indian featherback *Chitala chitala* from India (FJ459465.1), 95.03% (Pakistan, Bangladesh) and 93.75-94.05% to *C. lopis* from Kampar River, Riau (Indonesia) (KM213054.1) and Malaysia (KT001050.1). The COI is effectively used as species authentication method because intraspecific variation is low, but has high interspecific variation values especially in adjacent taxa [14].

3.2. Genetic Distance and Phylogenetic

Genetic distance at this study was also used to determine the genetic relationship between *species in Chitala genus* (Table 1).

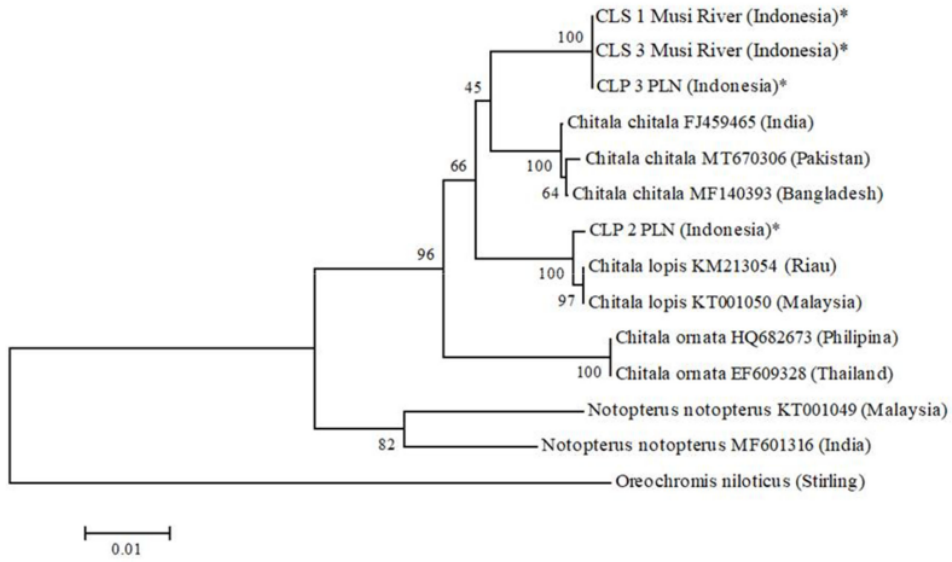
Table 1. Genetic distance between species in Chitala based on COI gene

No	Species	1	2	3	4	5	6	7	8	9	10	11	12	13
1	CLP 2 PLN (Indonesia)*													
2	*Chitala lopis* KT001050 (Malaysia)	0.006												
3	*Chitala lopis* KM213054 (Riau)	0.006	0.000											
4	*Chitala chitala* MF140393 (Bangladesh)	0.049	0.047	0.047										
5	*Chitala chitala* MT670306 (Pakistan)	0.049	0.047	0.047	0.004									
6	*Chitala chitala* FJ459465 (India)	0.047	0.046	0.046	0.002	0.006								
7	CLS 1 Musi River (Indonesia)*	0.059	0.061	0.061	0.044	0.047	0.042							
8	CLP 3 PLN (Indonesia)*	0.059	0.061	0.061	0.044	0.047	0.042	0.000						
9	CLS 3 Musi River (Indonesia)*	0.059	0.061	0.061	0.044	0.047	0.042	0.000	0.000					
10	*Chitala ornata* HQ682673 (Philippines)	0.076	0.070	0.070	0.070	0.074	0.068	0.072	0.072	0.072				
11	*Chitala ornata* EF609328 (Thailand)	0.076	0.070	0.070	0.070	0.074	0.068	0.072	0.072	0.072	0.000			
12	*Notopterus notopterus* MF601316 (India)	0.110	0.112	0.112	0.106	0.106	0.104	0.106	0.106	0.106	0.127	0.127		
13	*Notopterus notopterus* KT001049 (Malaysia)	0.118	0.116	0.116	0.118	0.118	0.120	0.123	0.123	0.123	0.135	0.135	0.074	
14	*Oreochromis niloticus* (Stirling)	0.226	0.231	0.231	0.228	0.228	0.230	0.220	0.220	0.220	0.224	0.224	0.228	0.218

* Specimen of current study

The genetic distance in Table 1 indicated that four samples of featherbacks were not 100% identical. CLP3, CLS1 and CLS 3, which is identified as *C. chitala* had a genetic distance of 0.06 (6%) to CLP2, which is annotated as *C. lopis*. The value of genetic distance within *C. lopis* based on the COI gene was 0-0.01, meanwhile within *C. chitala* ranged 0-0.04. Sequence differences of more than 5% can represent cryptic species, morphologically identical but different species [24]. The genetic distance of CLP2 samples was 0.01 (1%) with *Chitala lopis* from Malaysia (accession code KT001050.1) and from Sungai Kampar, Riau (KM213054.1). The smaller the genetic distance between individuals in a population, the more uniform the population will be. The table denoted that within the species barcode variation was low in compare to the sequence variation between *C. lopis, C. chitala* and *Notopterus notopterus*. The genetic distance between *C. chitala* and *N. notopterus* was 0.11-0.12, while *C. lopis* and *N. notopterus* varied from 0.11-0.12. This phenomenon can be said as a monophyletic relationship, which means a group of taxa originating from the same ancestor. Some studies also stated that the genetic distance within the genus is lower than between the genus [10,25].

The phylogenic tree of featherbacks was presented in Figure 1. This study determined the level of evolution and kinship of a species, where all Chitala were separated from *Notopterus* and species outgroup, *Oreochromis niloticus*.



* Specimen of current study

Figure 1. Phylogenetic tree of featherbacks from Musi River and Fishpond at PT PLN

The Figure 1 described that Chitala genus formed 4 sub-clusters. The first sub-cluster (bootstrap value/bv=100%) was consisted of samples from the Musi River (CLS1, CLS 3) and (CLP3) from PT PLN, meanwhile the second sub-cluster was *C. chitala* from Bangladesh, India and Pakistan. Although the two sub-clusters separate each other, the bootstrap value was quite low (bv=45%), therefore it cannot be stated as clearly distinct relative to the first sub cluster. The samples CLP2 (PT PLN), Malaysia and Kampar River (bootstrap value/bv = 100%) made third sub-cluster, identified as *C. lopis*. The fourth sub-cluster of Chitala genus was consisted of *C. ornate* from Thailand, Philippines and South Africa, formed a distant with high supported bootstrap value (98%) to others three sub cluster. The bootstrap in the phylogenetic tree was estimated to evaluate branch stability [26]. The bootstrap is fixed if the value is above 95% and it is unreliable if the bootstrap value is below 70%. Phylogenetic analysis of a species can be accomplished on morphological structure and genes through sequences of mitochondrial DNA. The mitochondrial DNA sequences elucidate the relationship of species to evolutionary complex due to variations in morphology [27]. The Chitala are experiencing decline population for many years due to over-exploitation and habitat destruction (4), therefore the need for the conservation of this genus are pivotal. The identification supported by DNA barcoding could be useful tool for appraising fish biodiversity and observing fish conservation (27–29).

The phylogenetic construction trees denoted clearly distant between species *C. lopis* and *C. chitala* (bootstrap value =70%). Another study stated that 7 out of 12 samples of featherbacks fish from Indragiri Hilir, Kampar, Musi and Mahakam River were identified as *C. lopis*. Meanwhile, the other 5 samples could not be classified with *C. lopis* because they had intraspecific variations of more than 3%. The existence of cryptic species in the featherbacks was showed with a large genetic variation in *C. lopis* group [28]. The utilization of DNA barcoding and morphology to delineate species may prove to be a solution for the appraisal of difficult cases such as cryptic species [9]. The second cluster was *N. notopterus*, consisted of 2 sub-clusters (bootstrap value of 82%), *N. notopterus* from Malaysia and

India. In *Notopterus*, there was strongly evidence of two allopatric species of *Notopterus,* which have been described from Southeast Asia and South Asia [21]. The third cluster was *Oreochromis niloticus* 1 species outgroup in this study. This research concordance with another study using allozyme, where genetic structure and relationships of the four species were phylogenetically separated as two clades, the *N. notopterus* and the clade of three *Chitala* species, revealing that the three *Chitala* species were genetically related more closely to each other than to *N. notopterus* [18].

4. Conclusions

The sample of featherbacks from PT PLN Indralaya (CLP2) indicated high similarity percentage to *C. lopis* originating from Malaysia (99.28%) and Kampar River, Riau (98.88%). Furthermore the Musi River (CLS1, CLS3) and PT PLN (CLP3) denoted a high percentage of similarities (95.53%) to *C. chitala* from India. The construction of phylogenetic trees indicated clearly distant between *C. lopis*, *C. chitala* and *Notopterus notopterus*., therefore DNA barcoding can be further applicable in the inventory and conservation of aquatic organism.

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