Phylogenetic Relationships of *Mystacoleucus marginatus* (Valenciennes 1842) based on Cytochrome Oxidase C Subunit I (COI) Gene

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**Abstract**  *Mystacoleucus marginatus* is a type of freshwater fish that is spread in various countries with tropical climates, one of which is Indonesia. This fish can be found in the Brantas River Basin (DAS), East Java. In its development, identification of fish species needs to be done in detail and more deeply, so that the correct identification results can be obtained. This study aims to identify genetic and phylogenetic characteristics of *Mystacoleucus marginatus* in the Brantas River Basin based on the Cytochrome Oxidase C Subunit I (COI) gene. Test samples were taken from 11 individuals of *Mystacoleucus marginatus* in the Brantas watershed, from June to August 2018. The results showed that *Mystacoleucus marginatus* in the Brantas watershed had 2 Haplotypes with Haplotype (Hd) = 0.96 diversity values and nucleotides diversity (Pi) = 0.00357. These results indicate that the population in the Brantas watershed is interconnected between one location and another sampling location, this is supported by a phylogenetic topology of DNA barcodes showing a monophyletic clade, as well as a haplotype distribution indicating that the sample area is genetics are not isolated from one another. So that the type of *Mystacoleucus marginatus* scattered along the Brantas watershed is still in one species, which is indicated by the low level of genetic variation. Genetic identification efforts must be carried out continuously so that appropriate information can be obtained for the diversity of fish species, especially endemic fish.

**Introduction**  
*Mystacoleucus marginatus* is a type of freshwater fish from the genus *Mystacoleucus* and the *Cyprinidae* family that live in the rivers of the tropics (Nelson, 2006; Froese & Pauly, 2014) and spread in Southeast Asia such as in Thailand (Rainboth, 2012; Vidthayanon, 2012) Myanmar (Kottelat, 2015), Laos (Pasco *et al.*, 2013), Cambodia (Kottelat, 2000; Kottelat 2001), and China (Huang *et al.*, 1979; Zheng *et al.*, 2017). Furthermore, this fish is also found throughout the Malay Peninsula (Meganathan *et al.*, 2015) to Indonesia (Sumatra and Java) (Dahruddin *et al.*, 2016), as well as in the upper part of the Kapuas lake in western Kalimantan, (Kottelat, 2013: Yang *et al.*, 2015).

One of the population areas of *Mystacoleucus marginatus* in Indonesia is in the Brantas River Basin (DAS) which is located in East Java and is one of the largest fisheries ecosystems and has several endemic fish species. Some fish that live in the Brantas watershed have not been identified molecularly and it is difficult to ascertain the correct name of each species. So it is necessary to identify species, especially molecularly, to confirm the naming of species (Pramono, 2017) and to know the genetic characteristics of the species so that it is easier to
assess the taxonomy of fish and provide useful information related to genetics in supporting the advancement of fisheries technology.

Along with the development of molecular biology, new methods have been found for the identification of DNA-based species known as DNA Barcoding (Hebert et al., 2003a). DNA barcoding provides speed and accuracy in species identification with a focus on analysis on small segments of mtDNA (Muchlisin et al., 2013; Karim et al., 2015). DNA barcoding can be a solution to the current taxonomic crisis (Meier et al., 2006). DNA-based identification techniques have also been used successfully to investigate genetic diversity (Ginneken et al., 2017), phylogenetic (Tang et al., 2011; Kusuma et al., 2016) and spatial connectivity between sub-populations and populations (Esa et al., 2008) from the Cyprinidae family.

Identification of genetic and phylogenetic diversity in this study was carried out using the cytochrome oxidase c subunit I (COI) gene which is a coding protein region of the mitochondrial genome (Hebert et al., 2003a; Liu et al., 2014). Globally, COI genes have been chosen as standard tools for molecular identification (Hebert et al., 2003a). Several molecular identification studies of Mystacoleucus marginatus with DNA Barcode application using COI genes have been successfully carried out (Pasco et al., 2013; Meganathan et al., 2015; Yang et al., 2015; Collins et al., 2016; Dahrudin et al., 2016; Zheng et al., 2016).

This study aimed to identify the genetic and phylogenetic characteristics of Mystacoleucus marginatus in the Brantas River Basin and its relationship to Mystacoleucus marginatus in other regions based on the cytochrome oxidase gene c subunit I (COI).

**Materials and methods**

**Sample collection**

In this study 11 test fish were taken from Malang, Blitar, Kediri and Sidoarjo. The four sampling locations are still in the Brantas River Basin area. Sampling was carried out in June-August 2018. For the purposes of molecular identification, a caudal fin was taken on each test fish and preserved in 95% ethanol solution.

**DNA extraction, isolation and amplification**

Genomic DNA extraction from all samples was carried out using the KIT method: Genomic DNA Mini Kit Animal Tissue (GENE AID). Amplification (PCR) of mitochondrial cytochrome C oxidase subunit I (COI) locus gene was carried out using GO TAQ Green PCR Mix method with universal primer pairs LCO1490: 5'-ggtcaacaatactaaatagtgttg3' and HCO2198: 5'-taactttcaggtgaccaaaaaatca-3' (Folmer et al., 1994) Mastermix (Go Taq Green) was made by adding ddH2O 14µL, LCOI and HCO2 2.5 µL, DMSO µL 1, 25 µL Go Taq Green and 5 µL DNA extraction, respectively. Amplification is carried out at a final volume of 50µl. The PCR process includes pre-denaturation at 94°C for 3 minutes, followed by 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and an extension stage at 72°C for 45 seconds. PCR results were then carried out by electroferesis process to separate, identify and purify DNA fragments, using 1% agarose gel with 50 mL Tris Borate EDTA (TBE). PCR results that have been successfully amplified are then sent to First Base CO (Malaysia) using Big Dye© terminator chemistry (Perkin Elmer), to get the basic arrangement that forms DNA or nucleotide sequences.

**Data analysis**

DNA sequencing data analysis was done by alignment and editing using MEGA 6.06 software (Tamura et al., 2011). Furthermore, sequence data is matched with data obtained from the NCBI (National Center for Biotechnology Information) genbank online (www.ncbi.nlm.gov) using the BLAST (Basic Local Alignment Search Tool) method. Nucleotide diversity analysis (𝜋), haplotype (h) and polymorphic DNA using DNASP 5.1 (Rozas et al.,
Phylogenetic reconstruction using the Maximum Likelihood Trees method (Lemey et al., 2009), Kimura-2 model parameter and 10000 × bootstrap value by using MEGA 6.06 software (Tamura et al., 2011). Haplotype network reconstruction using Network 5.0.

Results and discussions

Genetic characteristic

Figure 1. Electrophoretic Results of Mystacoleucus marginatus in the Brantas Watershed.

The length of the fragments resulting from the amplification of the Mystacoleucus marginatus COI gene obtained from the Brantas watershed using the primary LCO1490 and HCO2198 was 681-686 bp (base pairs) (Figure 1). The primary use of LCO1490 and HCO2198 was based on the research of Folmer et al., (1994), the results showed that the primary pair of LCO1490 and HCO2198 consistently strengthened the fragment of approximately 700 bp. This result is also consistent with the research conducted by Pasco et al. (2013); Meganatham (2015); Yang et al. (2015); Collins et al., (2016) Dahrudin et al., (2016) and Zheng et al., (2016) against Mystacoleucus marginatus using mitochondrial COI gene to produce sequences between 549-859 bp. Some differences in length of DNA fragments were amplified due to the type of primer used, primary basic composition, primary length, quality of DNA produced, food, offspring and environment (Shizuka & Lyon 2008). According to Hebert et al., (2003a), fragments along 658 bp using COI genes can be used as a basis for differentiating between animals.

All samples from the Brantas watershed were identified in Genbank using the BLAST method. The sample was identified as Mystacoleucus marginatus with a Query cover value between 95-99%, Identity values between 99-100% and E-value 0.0. Based on the results of BLAST analysis, it can be concluded that the DNA sequence of samples has a very high level of similarity with the DNA sequence in Genbank. Wahyuningsih (2014) argues that with 99-100% similarity level, it can be said that species are identical and can be identified as these species. Claverie and Notredame (2003) suggest that DNA sequences can be said to have homology if the E-value is smaller than e-0.4.
**Table 1. Mystacoleucus marginatus sequences**

| Sample code | Species outcome | Access code of NCBI | BLAST | Query Cover (%) | E-value | Identity (%) |
|-------------|-----------------|---------------------|-------|-----------------|---------|--------------|
| Malang¹     | M. marginatus   | KU692642.1          |       | 95              | 0.0     | 100          |
| Malang²     | M. marginatus   | KU692641.1          |       | 95              | 0.0     | 100          |
| Malang³     | M. marginatus   | KU692641.1          |       | 95              | 0.0     | 100          |
| Blitar¹     | M. marginatus   | KT001063.1          |       | 98              | 0.0     | 99           |
| Blitar²     | M. marginatus   | KT001063.1          |       | 97              | 0.0     | 99           |
| Blitar³     | M. marginatus   | KU692637.1          |       | 97              | 0.0     | 99           |
| Kediri¹     | M. marginatus   | KU692643.1          |       | 95              | 0.0     | 99           |
| Kediri²     | M. marginatus   | KU692639.1          |       | 95              | 0.0     | 99           |
| Kediri³     | M. marginatus   | KT001063.1          |       | 98              | 0.0     | 99           |
| Sidoarjo¹   | M. marginatus   | KU692637.1          |       | 99              | 0.0     | 99           |
| Sidoarjo²   | M. marginatus   | KU692636.1          |       | 95              | 0.0     | 99           |

**Remarks:** The identification of Mystacoleucus marginatus sequence through BLAST analysis.

**Genetic diversity**

The results of the analysis using the DnaSP 5.1 application found that Mystacoleucus marginatus in the Brantas watershed had a diversity of haplotypes (Hd)=0.96 and nucleotides diversity (Pi)=0.00357. The results showed eight nucleotide mutations, and seven polymorphic sites were identified. Hobbs et al., (2013) suggested that there are 2 categories of diversity, namely >0 hd <0.5, including in the low category and >0.5 hd <1 including into the high category. In addition, according to Nei (1987), the diversity of haplotypes (Hd) 0.1-0.4 belongs to the low category, (Hd) 0.5-0.7 medium category and (Hd) 0.8-2.00 high category. Based on this category, the value of the diversity of the Mystacoleucus marginatus haplotype in the Brantas watershed has a high level of diversity. The more diverse haplotypes, the higher the level of genetic diversity and vice versa (Fakhri et al., 2015).

The value of haplotype and nucleotide diversity in the Brantas watershed is higher than Malaysia with the Hd value; 0.5 and Pi; 0.00185, but lower than China, Hd; 1.00 and pi; 0.00357. This diversity can be determined by two factors: excessive exploitation and habitat conditions (Chiu et al., 2013). Nucleotides are DNA monomers that contain 3 different parts, namely pentose sugar, nitrogen bases (A, T, G, C) and phosphate groups (Toha, 2011). The average nucleotide composition found in the COI gene Mystacoleucus marginatus in the Brantas watershed was C (Cytosin) of 29.20%, T (Timin) of 27.04%, A (Adenine) of 27.70% and G (Guanin) of 16.01%. The whole sample of G + C is 45.24% and has a lower amount than the number of A + T which amounts to 54.76%, the low content of G + C makes it easier for us in the amplification process.

**Phylogeny and relatedness**

A phylogenetic tree or evolutionary tree is a mathematical structure that models evolutionary history based on the DNA sequence of a group of organisms (Page and Holmes 1998). Phylogenetic trees represent evolutionary distances between organisms. Phylogeny and relatedness of Mystacoleucus marginatus using a variation of nucleotide sequences on specific areas of mitochondrial genome (mtDNA) gene (COI) using the Maximum Likelihood (ML) method with 2-parameter Kimura model and 10000x bootstrap value in MEGA 6 software. Mystacoleucus marginatus from genbank as ingroup (positive control) and as outgroup are Tor tambroides and
Catostomus commersonii from Family *Cyprinidae*. The kinship relationship of *Mystacoleucus marginatus* from several regions can be seen in Fig. 2 as follows:

Phylogenetic trees form 7 branches. One branch has a value of 99 indicating that 10000x the 99% repetition will form the correct branching. According to Brinkman (2001), the branching of phylogenetic trees which is more than 70% is a branch that has truth with a 95% confidence interval. The results of the phylogenetic analysis of *Mystacoleucus marginatus* produce the same topology, differing only in the value of genetic diversity. This is due to the influence of Sundaland, where the Sundanese region has a shallow continental shelf and eustatic sea change repeatedly connects large islands in the region (Sundanese and Central Asian exposure) forming Sundaland (Rainboth, 1996; Voris, 2000). This allows the occurrence of freshwater migration into and outside of Java which may have occurred 10-70 thousand years ago (Kusuma, 2016).

From the Maximum Likelihood tree (ML) it can be concluded that *Mystacoleucus marginatus* is a monophyletic group. The monophyletic group of *Mystacoleucus marginatus* is divided into 2 main clades, namely clade A representing the population in the Brantas watershed (Malang, Blitar, Kediri, Sidoarjo and Mojokerto) and Malaysia, clade B representing the population of China. Clade A
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consists of 3 subclasses, namely A1 from the Brantas watershed (Malang, Blitar, Kediri, Sidoarjo and Mojokerto), A2 from Malang and A3 from the Malaysian population. Genetic distance (Table 4) subclade A1 (Malang, Blitar, Kediri, Mojokerto, Sidoarjo) by subclade A2 (Malang) which is 0.002, subclade A2 (Malang) with subclade A3 (Malaysia) which is 0.011 and the genetic distance of subclade A1 with A3 is 0.005. Clade B (China) has the largest genetic distance from the lowest value (Subclade A2; 0.056) to the highest value (Subclade A1: 0.090).

In general the genetic distance between clades is quite large but the distance between locations in the Brantas watershed (subclade A1: A2) is quite close. The Brantas watershed has a P-distance=0.000-0.002. Where 0.000 is the closest distance and this value indicates that of 1000 base pairs, none of them have different base pairs. This is because the Brantas watershed is still in one water-eyed waters on Mount Arjuno, the village of Brantas source, Kecamatan Bumi Aji, Kota Batu and then flows to Malang, Blitar, Kediri, Mojokerto and Sidoarjo. Current patterns like this result in geneflow or exchange of genes between locations resulting in homogeneous genes. This category can be included in the inbreeding category which results in an increase in the degree of homozygosity and at the same time decreases the degree of heterozygosity.

### Table 2. Genetic distance of Mystacoleucus marginatus.

|    | 1   | 2   | 3   | 4   | 5   | 6   |
|----|-----|-----|-----|-----|-----|-----|
| 1  | Malang |     |     |     |     |     |
| 2  | Blitar  | 0.002 |     |     |     |     |
| 3  | Kediri  | 0.002 | 0.000 |     |     |     |
| 4  | Sidoarjo | 0.002 | 0.000 | 0.000 |     |     |
| 5  | Mojokerto | 0.002 | 0.000 | 0.000 | 0.000 |     |
| 6  | Malaysia | 0.011 | 0.005 | 0.005 | 0.005 | 0.005 |
| 7  | China   | 0.090 | 0.074 | 0.074 | 0.074 | 0.074 |

Figure 3. The haplotype network of Mystacoleucus marginatus.
**Mystacoleucus marginatus** in the Brantas watershed is closely related between locations, from 15 individuals of the Brantas watershed (4 individuals obtained from genbank) 14 of them are in the same haplotype and one individual from Malang forms its own haplotype. The Malayian group forms two haplotypes and China forms 2 haplotypes. Between Brantas watershed groups, Malaysia and China are not related to each other. This is because there is no mixing of genes between locations in a long period of time because there is no more current connecting from when the location naturally.

**Conclusions**

**Mystacoleucus marginatus** in the Brantas watershed with a sequence of 681-686 bp (base pairs) has a diversity of haplotypes ($H_d$)=0.96 and nucleotides ($P_i$)=0.00357 shows a relationship with one another, this is supported by a 0.000- P-distance value 0.002 and phylogenetic topology shows a monophyletic clade, and haplotype distribution shows that the sample area is genetically not isolated from one another. Phylogenetic research on endemic fish must be carried out continuously, so that proper information is obtained regarding the presence of certain types of fish.

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