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DNA Barcoding: A Study of Guppy Fish (*Poecilia reticulata*) in East Java, Indonesia

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Abstract

Poecilia reticulata is a freshwater fish from the northeastern part of South America and spread widely to various countries in Asia and other continents. However, research about *P. reticulate* is limited even though it is a well-known fish species in Indonesia. The purpose of study was to identify the fish species of *P. reticulata* through DNA barcoding using the COI gene to determine the phylogenetic relationships among fish populations in East Java, Indonesia. In a present study, there were eight samples of *P. reticulata* from four different freshwater locations in East Java. Extraction, amplification, and sequencing of DNA samples were conducted to obtain the genetic data and construct a phylogenetic tree based on DNA sequences. The COI gene is the most popular markers to study genetic populations and phylogeography among the animal kingdom. Our phylogenetic reconstruction showed a clear that there were two groups of *P. reticulata*. The first group was obtain through species from East Java, Sukabumi, West Java (KU692776.1), Dominican Republic, Pandeglang, Banten and Myanmar. The second group was *P. reticulata* from southern Africa, Brazil, and Sukabumi, West Java (KU692775.1). The result of this study indicate that the guppy fish in East Java identic with *P. reticulata* from West Java (KU692776.1), which a widely used in classification based on evolutionary relationships. The findings of this study have important implication for the development of advance research about adaptation, phylogeny, and evolution of fish, especially of guppy fish.

How to Cite

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INTRODUCTION

The guppy (*Poecilia reticulata*) is a freshwater fish and a member of the Poecilidae family. Guppies are originated from the northeastern part of South America and have been introduced to many countries on every continent including Asia. Male guppies are smaller than the females. The males have a maximum length of 3.5 cm and the females are 6 cm in size. Female guppies have silvery colour with thin fins and larger than the males. Male guppies are polymorphisms. They have various combinations of colour patterns especially on the sides of the body and fins (Froese & Pauly, 2018). *P. reticulata* has several roles and benefits in life, including predators of several disease-causing mosquito larvae (Saleeza et al., 2014), used as ornamental aquarium fish (Singh et al., 2010), and act as an indicator of quality in the aquatic environments (Sarikaya et al., 2017).

There are 213 species of freshwater fish in the Java Island, Indonesia. Several species are endemic, but their ecosystem and biota are currently threatened (Hubert et al., 2015). In the Sunda area, the threatened biodiversity has increased over the past few centuries (Hoffman et al., 2010). The diversity and distribution of freshwater fish provide different data in the Java Island. Suryaningsih et al. (2018) revealed that *P. reticulata* can be found in the upper and middle parts of the river flow. *P. reticulata* is easily found in various area and widespread throughout the world (Deacon et al., 2011). *P. reticulata* can adapt even in polluted waters (Araujo et al., 2009), but research on genotypic variations related to environmental conditions is limited (Tezuka et al., 2011). The previous research with DNA barcoding demonstrated that genotypic variation of fish species in Java and Bali islands had a very large genetic distance even though in the same species (Dahruddin et al., 2016) and DNA barcoding of fin clip samples from fish can be used to biodiversity study in definite area and also in forensic analysis of a threatened wildlife (Nuryanto et al., 2018).

Molecular data is more widely used to make phylogenetic trees. It is because the data will be more stable in the evolutionary process compared to the morphological data (Dharmayanti, 2018). The activity of DNA barcoding based on fragments of the COI gene in the mitochondrial genome has been generally applied to identification and research of animal biodiversity including fish (Bingpeng, 2018). DNA barcoding can also be carried out to recognize species in terrestrial waters. Therefore, it can be used to monitor their distribution on the lake, river, and water ecosystems in Indonesia (Hubert et al., 2015). Species identification is essential for bio-conservation, preventing illegal exploitation, and protecting the species (Ciavaglia et al., 2015; Meganathan et al., 2013). However, study on *P. reticulata* is limited even though it spreads widely in Indonesia (Hubert et al., 2015).

The benefit of this investigation will help other researchers a new understanding of ecology, evolution, and classification on fish and especially of guppy fish. The purpose of the present study was to identify *P. reticulata* through DNA barcoding using the cytochrome c oxidase subunit I (COI) gene. It was expected to be useful to determine the phylogenetic relationship between *P. reticulata* populations in East Java, particularly in the river.

METHODS

Study Area and Sampling

The sampling process were conducted from January to February 2018. The fish were obtained from the freshwater river in Surabaya, Jombang, Malang, and Batu (Figure 1). Determination of sampling locations was performed based on the abundance of *P. reticulata* populations and their accessibility in the sampling process. The eight fish samples were obtained with 2 fish from each sampling location. Each sample was given a code based on the origin of the sample location (A1, A2, B1, B2, C1, C2, D1, and D2) (Table 1).

Figure 1. Sampling Location in four City or Regency, East Java.

DNA Extraction

The isolation, amplification, and observation process of DNA band sequencing was performed in the Molecular Genetic Laboratory of the Faculty of Science and Technology, Airlangga University, Surabaya. The DNA was isolated from muscle tissue or meat of fish using Jena

Table 1. Sampling locations

Sample Code	Sampling Location (City/ Regency)	Coordinate	
A1	Surabaya	7°16'36,1"LS	112°45'44,9"BT
A2			
B1	Jombang	7°26'24,1"LS	112°17'45,5"BT
B2			
C1	Malang	8°03'55,3"LS	112°37'48,4"BT
C2			
D1	Batu	7°51'54,0"LS	112°31'45,1"BT
D2			

Table 2. PCR materials

Material	Concentration	Volume (µL)
kit KAPA2G Fast ReadyMix	1x	24
Primer FishF1	0.5 Mm	2.5
Primer FishR1	0.5 Mm	2.5
ddH_2O	-	16
DNA sample	10-100 ng	2
Total	-	50

Table 3. PCR Condition

Step	Temperature (^{0}C)	Volume (µL)	Cycle
Pre-denaturation	96	3	1
Denaturation	96	0.5	40
Annealing	55	0.5	40
Extension	72	0.5	40
Post-extension	72	5	1

Bioscience reagent kit. It was performed using a column tube centrifugation method containing silicon to collect DNA from fish and clean up from the other impurities. DNA samples obtained from the isolation process can be directly used for DNA used for the next step, namely DNA amplification. If the isolated DNA sample is not used, it must be stored at -20°C.

DNA Amplification

DNA amplification was conducted by Polymerase Chain Reaction (PCR) method. It was done to obtain DNA from the COI gene. The copy of the DNA was performed using several materials and conditions according to Table 2 and Table 3. Therefore, the sequencing process can be done. After DNA amplification was carried out, electrophoresis was performed to examine the DNA samples and the base pairs (bp). The amplified target DNA was from the mitochondrial COI gene with a base length of around 600 bp.

DNA Sequencing

DNA samples with a pair of FishF1 and FishR1 primer were delivered to First BASE Laboratory through Genetics Science Indonesia Company, Jakarta, Indonesia. Data from DNA band sequencing was obtained within two weeks. The results of DNA nucleotide bases (A, T, G, and C) along with graphs of sequential chromatograms were obtained through the website of download.base-asia.com.

Data Analysis

Forward and Reverse sequencing were performed to obtain DNA sequences. Then, trimming process was performed. MEGA6 software was used to combine a pair of DNA sequences in order to produce a nucleotide base sequence from each sample. Basic Local Alignment Search Tool (BLAST) analysis was conducted by using a nucleotide bases sequence. BLAST analysis was

performed to examine the genetic species from each sample. It was obtained through alignment with data on the nucleotide base sequence from GenBank data. MEGA6 software was also used to compile phylogenetic trees based on the DNA bands sequence for each sample. Phylogenetic trees were made by using sequence data from this study and GenBank. The Neighbor-Joining Tree method with Bootstrap 1000 times was used to make the phylogenetic trees.

RESULTS AND DISCUSSION

A pair of primers will flank the desired sequence area on the DNA sample for amplification. DNA polymerase acts to compile a new DNA band based on the area flanked by a pair of primer. The mixture of the primer ingredients, nucleotides, and DNA polymerase will be able to react in the PCR machine (thermal cycler). It can carry out heating and cooling cycles automatically. Each cycle takes several minutes. PCR generates billions copies of DNA band. DNA samples can be useful to analyze various purposes (Audesirk, 2012).

In the present study, eight samples of *P. reticulata* were utilized for observation. The amplification results of A1, A2, B1, B2, C1, C2, D1, and D2 demonstrated a visible band with a base length between 500 - 750 bp (Figure 2). The bands of A1 and A2 samples were more visible than bands of B1, B2, C1, C2, D1, and D2 (Figure 3). According to Lee et al. (2002), the distinct of DNA band thickness indicated the distinct of DNA concentrations. The higher DNA concentration indicated the more visible of DNA band. It revealed that A1 and A2 samples had higher DNA concentrations compared to B1, B2, C1, C2, D1, and D2 sample. DNA bands on gel electrophoresis that have more extensive base lengths will migrate slowly from the negative pole to the positive pole, while DNA bands that have smaller base lengths can migrate more quickly (Lee et al., 2002).

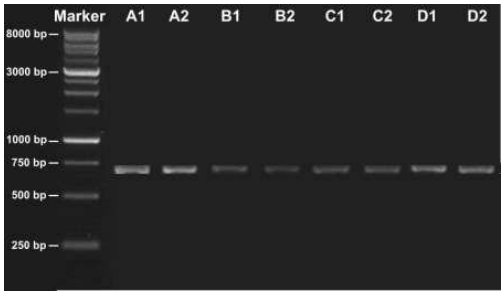


Figure 2. DNA electrophoresis result of COI gene

Fish F1 and Fish R1 primers were used to determine the length of PCR amplification fragments. The result of PCR amplification with the COI gene, Fish F1 and Fish R1 primers demonstrated that each sample had more than 500 bp in size (Figure 3). According to Hebert et al. (2003), barcoding COI gene should be 648 bp in length. Sequences of COI genes are larger than 500 bp on the edge of the 5 'COI gene with sufficient information can be categorized in GenBank as DNA barcodes (Benson et al., 2005). DNA barcoding is useful to identify a species by comparing the DNA nucleotide (nitrogen base) sequence to the same gene from other known species. In addition, DNA barcoding has been widely used for identifying the taxonomic status of a species but not among individuals in the same species. This approach has proven to be useful in animal kingdom when using parts of the mitochondrial COI gene (CBOL, 2009). The mitochondrial COI gene is the most popular markers for the study of genetic populations and phylogeography among the animal kingdom. The COI gene has high base nitrogen of Adenosine and Thymine and high level of nucleotide variation. COI gene also can be used for the identification of marine nematode species (Derycke et al., 2010) and fish species (Chang et al., 2016).

In the present study, DNA sequences from *P. reticulata* in East Java and sample sequences from GenBank's, were combined to compile phylogeny trees. There were two groups of *P. reticulate,* which were formed from 18 samples of *P. reticulata* and one species of *Micropoecilia picta* used as out groups. The first group was obtained from *P. reticulata* species in East Java (A1, A2, B1, B2, C1, C2, D1, and D2); Sukabumi, West Java (KU692776.1); Dominican Republic (JX968694.1); Pandeglang, Banten (KU692774.1); and Myanmar (LC190039.1 and LC190038.1), while the second group was obtained from southern Africa (KU568970.1 and KU568971.1); Brazil (GU702150.1 and GU702152.1); and Sukabumi, West Java (KU692775.1) (Figure 3). There are two groups of *P. reticulata* because they live in a different environment even though they are from the same species. Therefore, it urgently needs to investigate the second group. Phylogenetic are the relationship based on the composition of DNA or protein sequences that are similar to examine the evolutionary process (Baldauf, 2003). The phylogeny tree provides information about population classification based on evolutionary relationships.

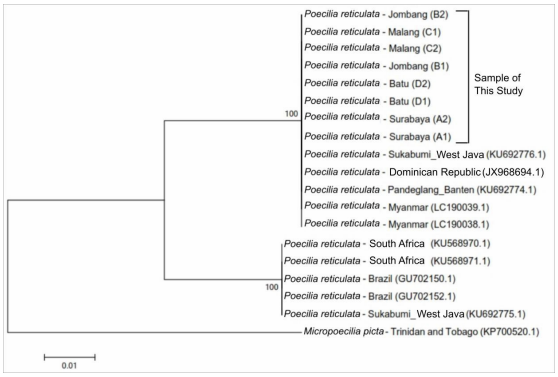
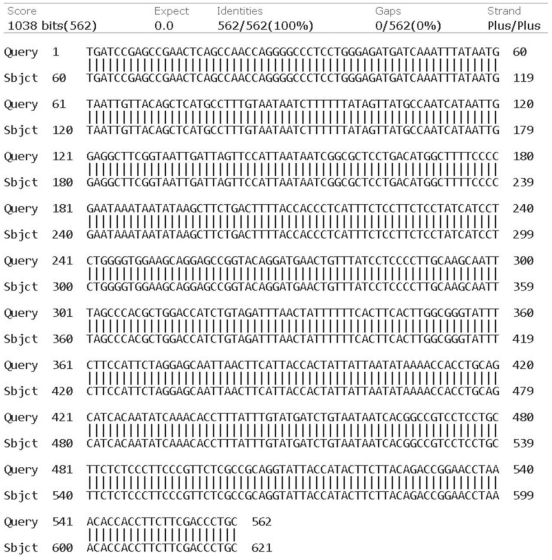


Figure 3. Phylogeny trees based on DNA sequences along with secondary data from Gen Bank (species name followed by origin area and sample code)

Guppy fish *(P. reticulata)* studied in this research (Surabaya, Jombang, Malang, and Batu) was in one group with *P. reticulata* species from the Sukabumi area, West Java (KU692776.1), Dominican Republic (JX968694.1), Pabdeglang, Banten (KU692774.1), and Myanmar (LC190039.1 and LC190038.1). However, they are separated from the second group for those from southern Africa (KU568970.1 and KU568971.1); Brazil (GU702150.1 and GU702152.1); and Sukabumi, West Java (KU692775.1) because they have a very identical sequence of nucleotide bases of 100% (Figure 4).

P. reticulata studied in this study was separate from the *P. reticulata* group originating from southern Africa (KU568970.1 and KU568971.1); Brazil (GU702150.1 and GU702152.1); and Sukabumi, West Java (KU692775.1) because they only have a lower level of similarity, which is 95% among nucleotide base sequences. There are 27 different nucleotide bases between the 2 groups of *P. reticulata* after the analysis (Figure 5). Previous research conducted by Dahruddin et al. (2016) showed that the *P. reticulata* group had a substantial genetic distance even in similar species with a value difference of 4.77%. The introduction of new species and hybridization among descendants in different populations increase the genetic variation (Kolbe et al., 2004), and the introduction of new species can construct a new genotypes (Ellstrand & Schierenbeck, 2000), and disguise adverse mutations (Loewe & Hill, 2010). Tarallo et al. (2016) revealed that salinity and migration affect not only the physiological and morphological characters but also the genes character (nucleotide base consist of G and C) of teleost. These factors increase the invasion and adaptation to new areas (Perry et al., 2001). DNA barcoding has been widely used to identify a gene species by comparing nucleotide sequences. The mitochondrial of COI gene is the most popular markers to study genetic populations and phylogeography, particularly in fish. Phylogenetic is the relationship based on identical DNA or protein sequence composition to estimate the evolutionary process and evolutionary relationships of living things.



Score	Expect	Identities	Gaps	Strand
1038 bits(562)	0.0	562/562(100%)	0/562(0%)	Plus/Plus

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Query  1    TGATCCGAGCCGAACTCAGCCAACCAGGGGCCCTCCTGGGAGATGATCAAATTTATAATG  60
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Query  61   TAATTGTTACAGCTCATGCCTTTGTAATAATCTTTTTTATAGTTATGCCAATCATAATTG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  120  TAATTGTTACAGCTCATGCCTTTGTAATAATCTTTTTTATAGTTATGCCAATCATAATTG  179

Query  121  GAGGCTTCGGTAATTGATTAGTTCCATTAATAATCGGCGCTCCTGACATGGCTTTTCCCC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180  GAGGCTTCGGTAATTGATTAGTTCCATTAATAATCGGCGCTCCTGACATGGCTTTTCCCC  239

Query  181  GAATAAATAATATAAGCTTCTGACTTTTACCACCCTCATTTCTCCTTCTCCTATCATCCT  240
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Sbjct  240  GAATAAATAATATAAGCTTCTGACTTTTACCACCCTCATTTCTCCTTCTCCTATCATCCT  299

Query  241  CTGGGGTGGAAGCAGGAGCCGGTACAGGATGAACTGTTTATCCTCCCCTTGCAAGCAATT  300
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Query  361  CTTCCATTCTAGGAGCAATTAACTTCATTACCACTATTATTAATATAAAACCACCTGCAG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  421  CATCACAATATCAAACACCTTTATTTGTATGATCTGTAATAATCACGGCCGTCCTCCTGC  480
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Query  541  ACACCACCTTCTTCGACCCTGC  562
            |||||||||||||||||||||||
Sbjct  600  ACACCACCTTCTTCGACCCTGC  621
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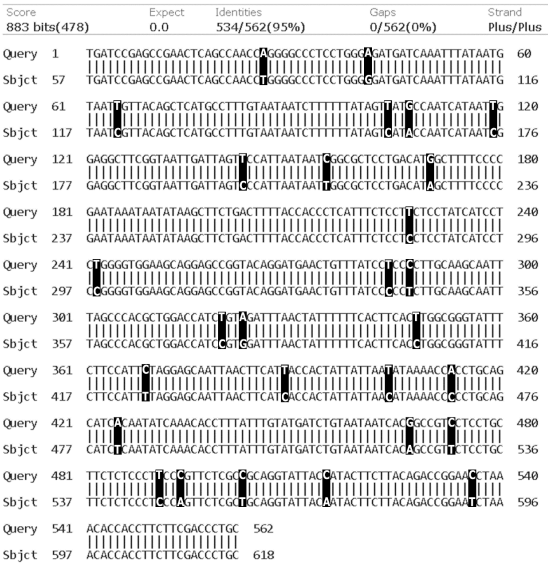
Figure 4. The sequences of nitrogen DNA base is identic between the sample of this study and other research samples



Score	Expect	Identities	Gaps	Strand
883 bits(478)	0.0	534/562(95%)	0/562(0%)	Plus/Plus

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Query  1    TGATCCGAGCCGAACTCAGCCAACCAGGGGCCCTCCTGGGAGATGATCAAATTTATAATG  60
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Query  241  CTGGGGTGGAAGCAGGAGCCGGTACAGGATGAACTGTTTATCCTCCCCTTGCAAGCAATT  300
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Query  301  TAGCCCACGCTGGACCATCTGTAGATTTAACTATTTTTTCACTTCACTTGGCGGGTATTT  360
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Query  541  ACACCACCTTCTTCGACCCTGC  562
            ||||||||||||||||||||||
Sbjct  597  ACACCACCTTCTTCGACCCTGC  618
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Figure 5. The different of DNA sequences between the sample of this study and other research samples

These results of this research serve valuable data about the genotype of fish, especially genotype of species guppy fish in East Java. In addition, data from this study is also important

for further advance research of adaptation, phylogeny, and evolution of fish,

CONCLUSION

There is a relationship between *P. reticulata* species in East Java from Surabaya, Jombang, Malang and Batu. They are identical and are in the same group in the phylogenetic tree. *P. reticulata* from East Java is also identical and is in the same phylogenetic group with species from other regions such as Sukabumi, West Java (KU692776.1); Pandeglang, Banten; Dominican Republic; and Myanmar even though they are genetically different and placed in different group from *P. reticulata* from the South African; Brazil; and Sukabumi, West Java (KU692775.1).

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