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DNA Barcoding: A Study of Guppy Fish (Poecilia reticulata) in East Java, Indonesia Sucipto Hariyanto, Hasan Adro'i, Mahrus Ali, Bambang Irawan

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REVIEWER RECOMMENDATIONS AND COMMENTS & RESPONSES TO REVIEWER COMMENTS

DNA Barcoding: A Study of Guppy Fish (*Poecilia reticulata*) in East Java, Indonesia ~~Based on Cytochrome c Oxidase Subunit I (COI)~~ [U1]

Sucipto Hariyanto* , Hasan Adro'i, Mahrus Ali, and Bambang Irawan

Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya, 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. The fish was introduced in 1920 as a biological control agent and has developed through natural reproduction. The fish has several roles as predator of several mosquito larvae, ornamental fish, and indicators of the quality environment. Several studies on the diversity and distribution of freshwater fishes on Java have different data. The fish found easily live in environments with various conditions. The fish has high adaptable ability even in polluted waters. [U2]

The purpose of study is to identify of fish species *P. reticulata* through DNA barcoding using the COI gene to determine phylogenetic relationships among fish populations in East Java, Indonesia. Research about *P. reticulate* is limited even though it is well-known fish species in 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 phylogeny tree based on DNA sequences. The COI gene is the most popular markers to study genetic populations and phylogeography among the animal kingdom. There were two groups of *P. reticulata* for phylogeny tree. The first group was obtained through species samples 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 phylogeny tree provides information about population classification based on evolutionary relationships.

Explain the novelty and Implication or Benefit your research for science development/society here…….(±2 lines)

Keywords: *Poecilia reticulata*, DNA barcoding, COI gene, phylogeny

INTRODUCTION

The guppy (*Poecilia reticulata*) is a freshwater fish and member of the family Poecilidae. 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 female guppies. Male guppies have a maximum length of 3.5 cm and females are 6 cm in size. Female guppies have silvery colour with thin fins and larger than males. Male guppies are polymorphisms. They have various

combinations of colour patterns especially on the sides of the body and fins (Froese & Pauly, 2018). In Indonesia, the guppy (*Poecilia reticulate*) was introduced in 1920 as biological control agents and has developed through natural reproduction in environments (Eidman,1989[U3]). *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 spesies of freshwater fish in the Java Island, Indonesia. Several spesies are endemic, but the ecosystem and biota are currently threatened (Hubert *et al*., 2015). In the Sunda area, the biodiversity threat 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). 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 (Nuryanto *et al*., 2018).

~~The purpose of the present study is to identify *P. reticulata* through DNA barcoding using the cytochrome c oxidase subunit I (COI) gene. It is useful to determine the phylogenetic relationship between *P. reticulata* populations in East Java, particularly in the river.~~

[U4]Molecular data is more widely used to make phylogenetic trees. It due to data will be more stable in the evolutionary process compared to morphological data (Dharmayanti, 2018). The activity of DNA barcoding based on fragments of the COI gene. It found in mitochondrial organelles and 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 the 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 species (Ciavaglia *et al.*,2015; Meganathan *et al*., 2013). *P. reticulata* research is limited even tough it spreads widely in Indonesia (Hubert *et al*., 2015).

The purpose of the present study is to identify *P. reticulata* through DNA barcoding using the cytochrome c oxidase subunit I (COI) gene. It is useful to determine the phylogenetic

relationship between *P. reticulata* populations in East Java, particularly in the river. [U5]

METHODS

Study Area and Sampling

The samples were conducted from January to February 2018. Fish was obtain 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 access in the sampling process. The eight fish samples was obtained with 2 fish from each sampling location. It was performed to DNA analysis. 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).

TABLE 1. Sampling locations

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

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 isolation process was obtained from muscle tissue or meat of fish using Jena Bioscience reagent kit. It was performed using a column tube centrifugation method containing silicon to collect DNA from fish and clean up from other impurities. DNA samples obtained from the isolation process can be directly used for DNA

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 perfomed to examine the DNA samples and the base length (bp). The target DNA amplified was from the base length mitochondrial COI gene around 600 bp (electro image).

TABLE 2. PCR materials

	Material	Concentration	Volume (µL)
1	kit *KAPA2G Fast ReadyMix*	1X	24
2	Primer FishF1	0,5 [U6]Mm	2,5
3	Primer FishR1	0,5 Mm	2,5
4	ddH_2O	-	16
5	DNA sample	10-100 ng	2
6	Total	-	50

TABLE 3. PCR Condition

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

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 Gene bank data. MEGA6 software was also used to compile phylogenetic trees based on the DNA bands sequence for each sample. Phylogeny trees were made by using sequence data from this study and Gene bank. The Neighbor-Joining Tree method with Bootstrap 1000 times was used to make phylogeny 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 generate billions of 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 sample 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).

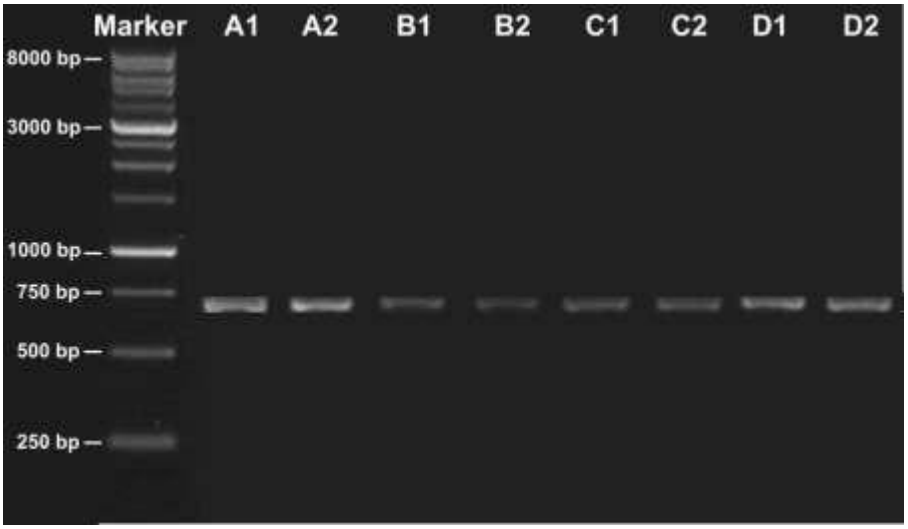


Figure 2. DNA electrophoresis result of COI gene

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) suggested that 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 of 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 identification of fish species (Chang *et al*., 2016).

In the present study, DNA sequences from *P. reticulata* in East Java and sample sequences from Gene Bank'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*. They were 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 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. In the reconstruction of phylogenetic trees, molecular data is more widely used due to it is considered more stable in the evolutionary process compared to morphological data (Dharmayanti, 2011).

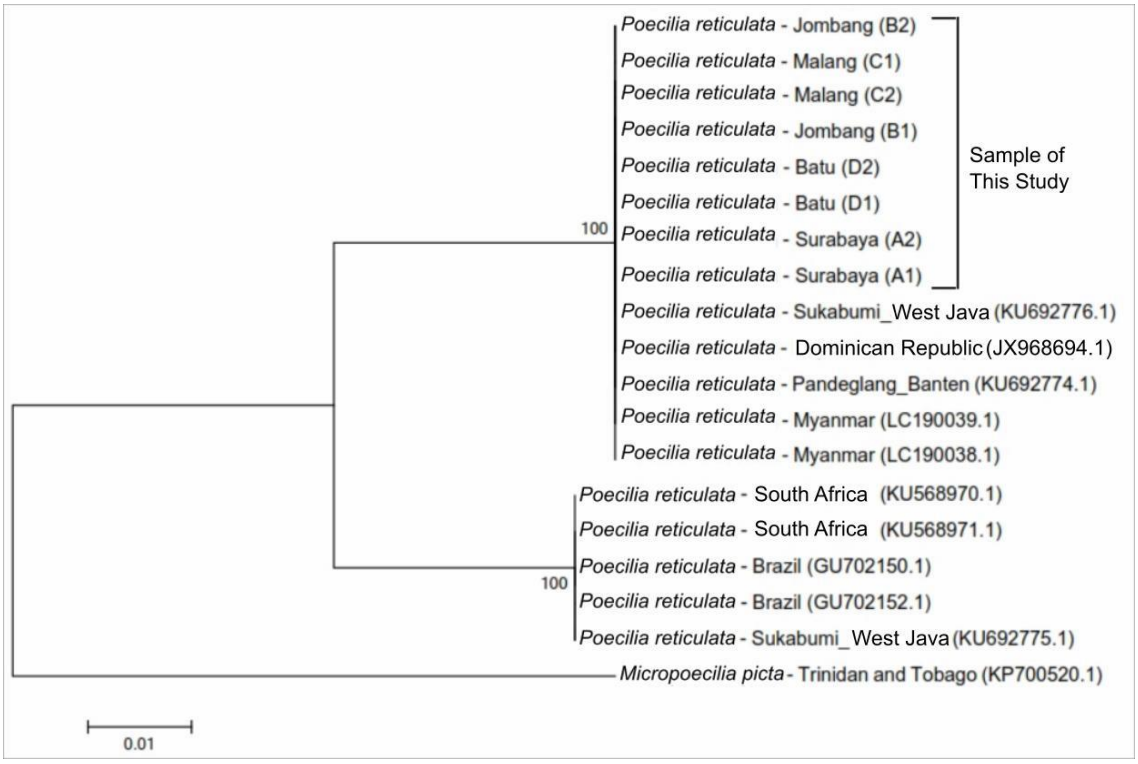
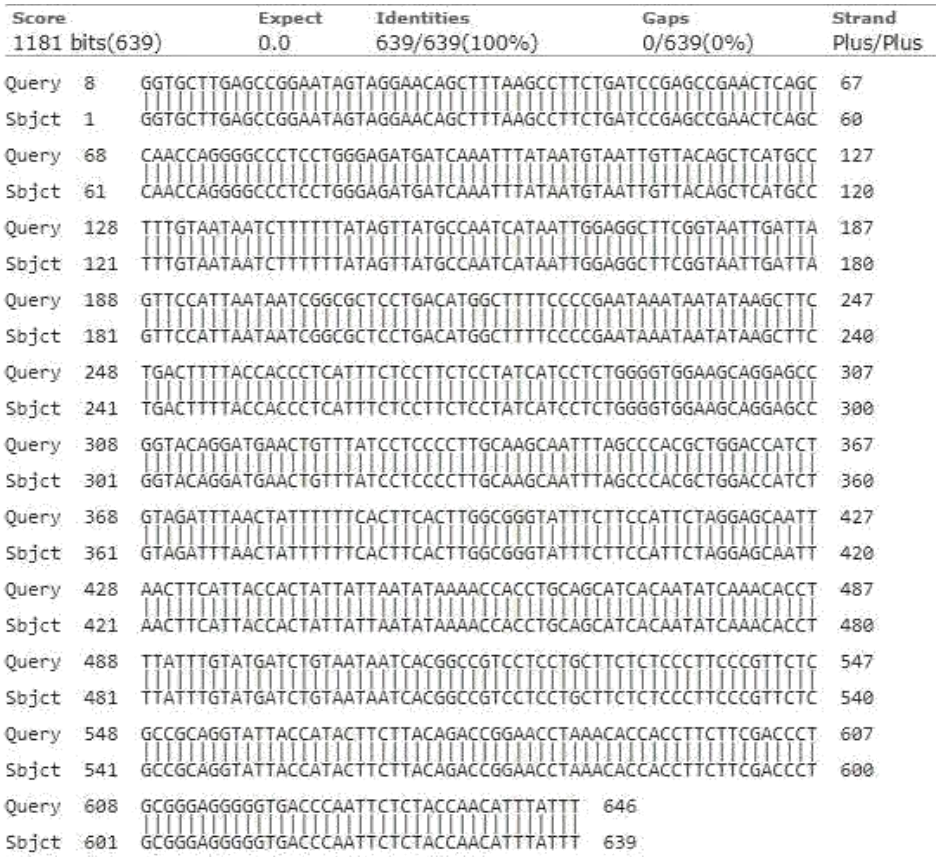


Figure 3. Phylogeny trees based on DNA sequences along with secondary data from Gene bank (species name followed by origin area and sample code)

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 separate from the second group namely 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 in 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 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 genetic variation (Kolbe *et al*., 2004), construct a new genotypes (Ellstrand & Schierenbeck, 2000), and disguise adverse mutations (Riescberg *et al*., 1999). Tarallo *et al.* (2016) revealed that salinity and migration can't impact on physiological and morphological characters but also the genes character (nucleotide base consist of G and C) in fish (Tarallo *et al.*, 2016). These factors increase 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. Phylogenetics 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
1181 bits(639)	0.0	639/639(100%)	0/639(0%)	Plus/Plus

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Query  8    GGTGCTTGAGCCGGAATAGTAGGAACAGCTTTAAGCCTTCTGATCCGAGCCGAACTCAGC  67
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GGTGCTTGAGCCGGAATAGTAGGAACAGCTTTAAGCCTTCTGATCCGAGCCGAACTCAGC  60

Query  68   CAACCAGGGGCCCTCCTGGGAGATGATCAAATTTATAATGTAATTGTTACAGCTCATGCC  127
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CAACCAGGGGCCCTCCTGGGAGATGATCAAATTTATAATGTAATTGTTACAGCTCATGCC  120

Query  128  TTTGTAATAATCTTTTTTATAGTTATGCCAATCATAATTGGAGGCTTCGGTAATTGATTA  187
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  TGACTTTTACCACCCTCATTTCTCCTTCTCCTATCATCCTCTGGGGTGGAAGCAGGAGCC  300

Query  308  GGTACAGGATGAACTGTTTATCCTCCCCTTGCAAGCAATTTAGCCCACGCTGGACCATCT  367
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  368  GTAGATTTAACTATTTTTTCACTTCACTTGGCGGGTATTTCTTCCATTCTAGGAGCAATT  427
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GTAGATTTAACTATTTTTTCACTTCACTTGGCGGGTATTTCTTCCATTCTAGGAGCAATT  420

Query  428  AACTTCATTACCACTATTATTAATATAAAACCACCTGCAGCATCACAATATCAAACACCT  487
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Sbjct  421  AACTTCATTACCACTATTATTAATATAAAACCACCTGCAGCATCACAATATCAAACACCT  480

Query  488  TTATTTGTATGATCTGTAATAATCACGGCCGTCCTCCTGCTTCTCTCCCTTCCCGTTCTC  547
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  608  GCGGGAGGGGGTGACCCAATTCTCTACCAACATTTATTT  646
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Figure 4. The sequences of nitrogen DNA base is identic between the sample of this study and other research samples

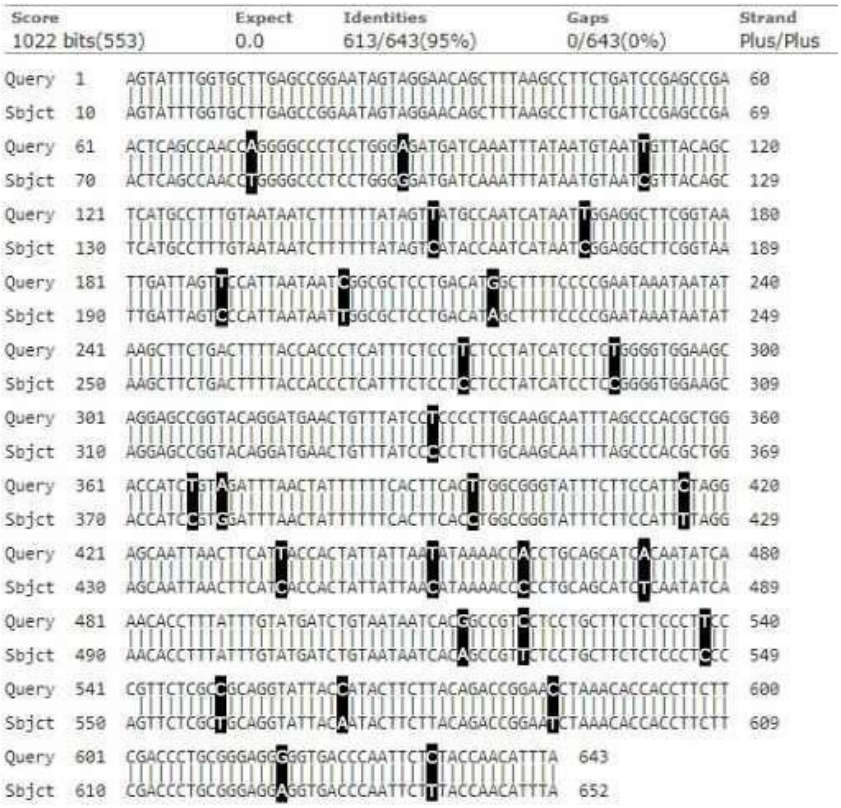

Figure 5. The different of DNA sequences between the sample of this study and other research samples

For Closing statement :

- Explain the novelty of your research and
- The benefits and contribution of research for the science/ society

CONCLUSIONS

The relationship between *P. reticulata* species in East Java from Surabaya, Jombang, Malang and Batu was identical and had the same group in the phylogeny tree. *P. reticulata* from East Java was identical and had a phylogeny group with species from other regions such as Sukabumi, West Java (KU692776.1); Pandeglang, Banten; Dominican Republic; and Myanmar even though they were genetically different and had different group of *P. reticulata* from the South African; Brazil; and Sukabumi, West Java (KU692775.1).

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Table list of revision article DNA Barcoding (Hariyanto et al)

August 18 2019

Page	Line	Suggestion of reviewer	Author remarks
1	21-24	Complete or rewrite in more appropriate structure to provide a clear and correct information	Rewrite (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).
1	24-26	Do provide the study results appropriately	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.
2	10-12	Complete or rewrite this past in more appropriate structure to provide a clear and correct information	…. 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).
2	14-15	Complete this sentence appropriately to provide a clear and correct information and do provide the citation	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).
2	15-17	Complete this sentence appropriately to provide a clear and correct information and do provide the citation. Do provide the paraphrase appropriately.	
2	25-27	Move t the end of Introduction	I am agree
3	5	Do provide some sentences showing the benefit expected from this study to the science development or society.	I have written
3	13-14	Rewrite in more appropriate structure to provide a clear and correct information	I have rewrite
		Correct this one	I correct it
7	6-7	This sentence has been stated in introduction.	deleted
		Correct this one	I have already revised it

		All references are not written in APA style correctly. Correct all references, pay attention to details	
12	16	The year is too old , please change with the 10 year latest	I am agree deleted
12	17	Not found in body text. Add the in-text citation or remove	I am agree remove

DNA Barcoding: A Study of Guppy Fish (*Poecilia reticulata*) in East Java, Indonesia ~~Based on Cytochrome c Oxidase Subunit I (CO~~

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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, r~~R~~esearch about *P. reticulate* is limited even though it is a well-known fish species in Indonesia. The purpose of study ~~is~~ was to identify ~~of~~ 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. ~~Research about *P. reticulate* is limited even though it is well-known fish species in 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 ~~phylogeny~~ phylogenetic tree based on DNA sequences. The COI gene is the most popular markers to study genetic populations and phylogeography among the animal kingdom. There were two groups of *P. reticulata* for phylogeny tree. The first group was obtained through species samples 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).[M1] **RESULTS**[M2] The ~~phylogeny~~ phylogenetic tree provides information about population classification based on evolutionary relationships.
The~~se~~ findings of this study have important implication for the development ~~developing for~~of advance research about adaptation, phylogeny, and evolution of fish, especially of guppy fish.

Keywords: *Poecilia reticulata*, DNA barcoding, COI gene, phylogeny

INTRODUCTION

The guppy (*Poecilia reticulata*) is a freshwater fish and a member of the ~~family~~ 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 female~~s guppies~~. The ~~M~~males ~~guppies~~ 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 spec~~s~~ies of freshwater fish in the Java Island, Indonesia. Several spec~~s~~ies are endemic, but their ecosystem and biota are currently threatened (Hubert et al., 2015). In the Sunda area, the threatened biodiversity ~~threat~~ 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 ~~P~~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 [M3](Nuryanto et al., 2018).

~~The purpose of the present study is to identify *P. reticulata* through DNA barcoding using the cytochrome c oxidase subunit I (COI) gene. It is useful to determine the phylogenetic relationship between *P. reticulata* populations in East Java, particularly in the river.~~Molecular data is more widely used to make phylogenetic trees. It ~~due to~~ 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[M4]. It found in mitochondrial organelles and has been generally applied to identification and research of animal biodiversity including fish[M5] (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* ~~research~~ is limited even though it spreads widely in Indonesia (Hubert et al., 2015).

The purpose of the present study ~~is~~ was to identify *P. reticulata* through DNA barcoding using the cytochrome c oxidase subunit I (COI) gene. It ~~is~~ was expected to be useful to determine the phylogenetic relationship between *P. reticulata* populations in East Java, particularly in the river. [U6]

BENEFIT[M7]

METHODS

Study Area and Sampling

The ~~samples~~ sampling process were conducted from January to February 2018. The ~~F~~fish ~~was~~ 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 ~~was~~ were obtained with 2 fish from each sampling location. ~~It was performed to DNA analysis.~~ 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).

TABLE 1. Sampling locations

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

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 ~~isolation process~~ was ~~obtained~~ isolated from muscle tissue or meat of fish using Jena 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[M8]

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 perfo rmed to examine the DNA samples and the base ~~length~~ pairs (bp). The amplified target DNA ~~amplified~~ was from the base ~~length~~ pairs of mitochondrial COI gene was around 600 bp (electro image).[M9]

TABLE 2. PCR materials

	Material	Concentration	Volume (µL)
1	kit *KAPA2G Fast ReadyMix*	1x	24
2	Primer FishF1	0.5 [U10]Mm	2.5
3	Primer FishR1	0.5 Mm	2.5
4	ddH_2O	-	16
5	DNA sample	10-100 ng	2
6	Total	-	50

TABLE 3. PCR Condition

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

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 Gen~~e b~~Bank data. MEGA6 software was also used to compile phylogenetic trees based on the DNA bands sequence for each sample. Phylogenetic~~y~~ trees were made by using sequence data from this study and GenBe~~ b~~ank. The Neighbor-Joining Tree method with Bootstrap 1000 times was used to make the phylogenetic~~y~~ 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 ~~of~~ 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).

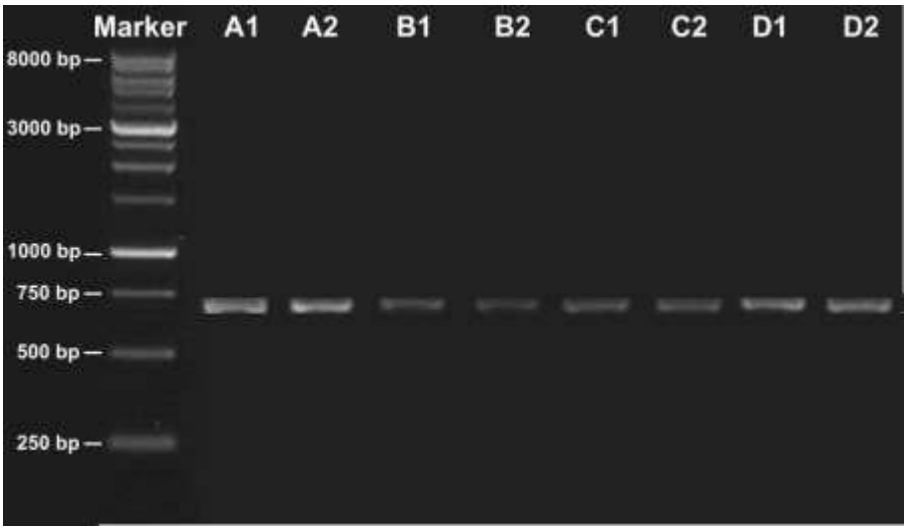

Figure 2. DNA electrophoresis result of COI gene

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), ~~suggested that~~ 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 ~~of~~ 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 ~~identification of~~ fish species (Chang et al., 2016).

In the present study, DNA sequences from *P. reticulata* in East Java and sample sequences from Gene Bank'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*. They were used as out groups. [M11]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. In the reconstruction of phylogenetic trees, molecular data is more widely used due to it is considered more stable in the evolutionary process compared to morphological data (Dharmayanti, 2011).[M12]

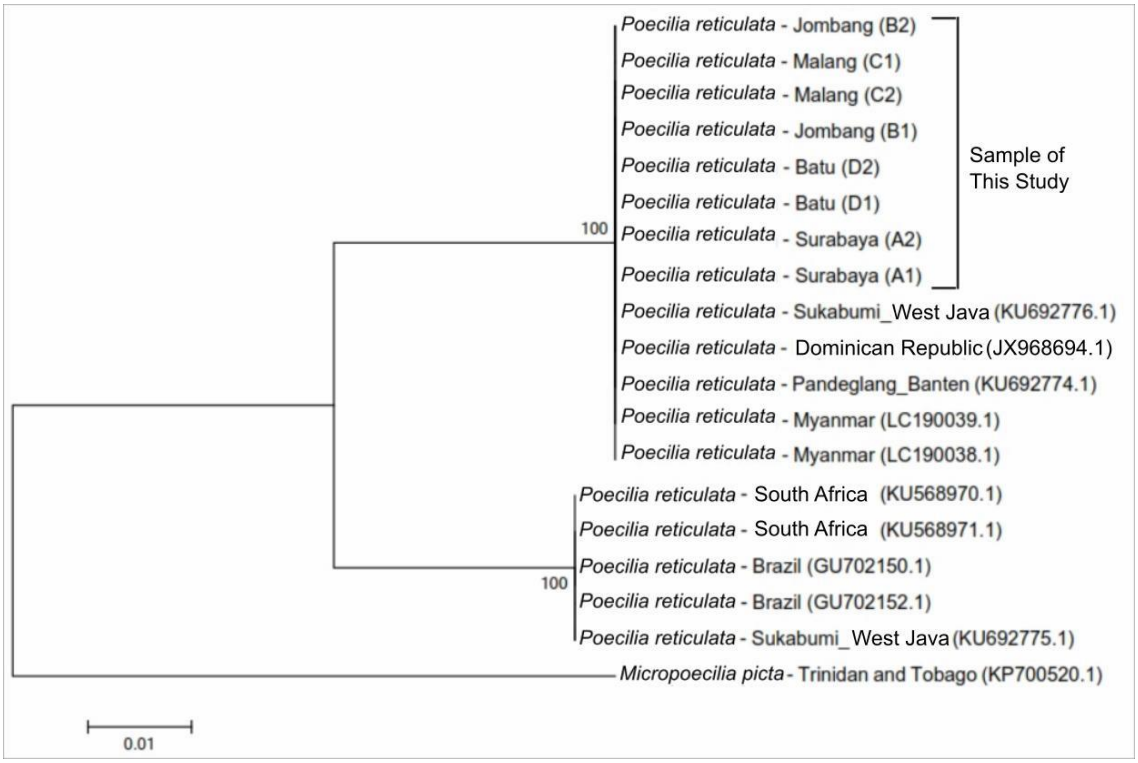

Figure 3. Phylogeny trees based on DNA sequences along with secondary data from Gene bBank (species name followed by origin area and sample code)

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 ~~namely~~ 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 ~~in~~ 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), construct a new genotypes [M13](Ellstrand & Schierenbeck, 2000), and disguise adverse mutations (Loewe & Hill, 2010). Tarallo et al. (2016) revealed that salinity and migration can't ~~impact~~ affect the~~on~~ physiological and morphological characters but also the genes character[M14] (nucleotide base consist of G and C) in fish (Tarallo et al., 2016). 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~~s~~ is the relationship based on identical DNA or protein sequence composition to estimate the evolutionary process and evolutionary relationships of living things.

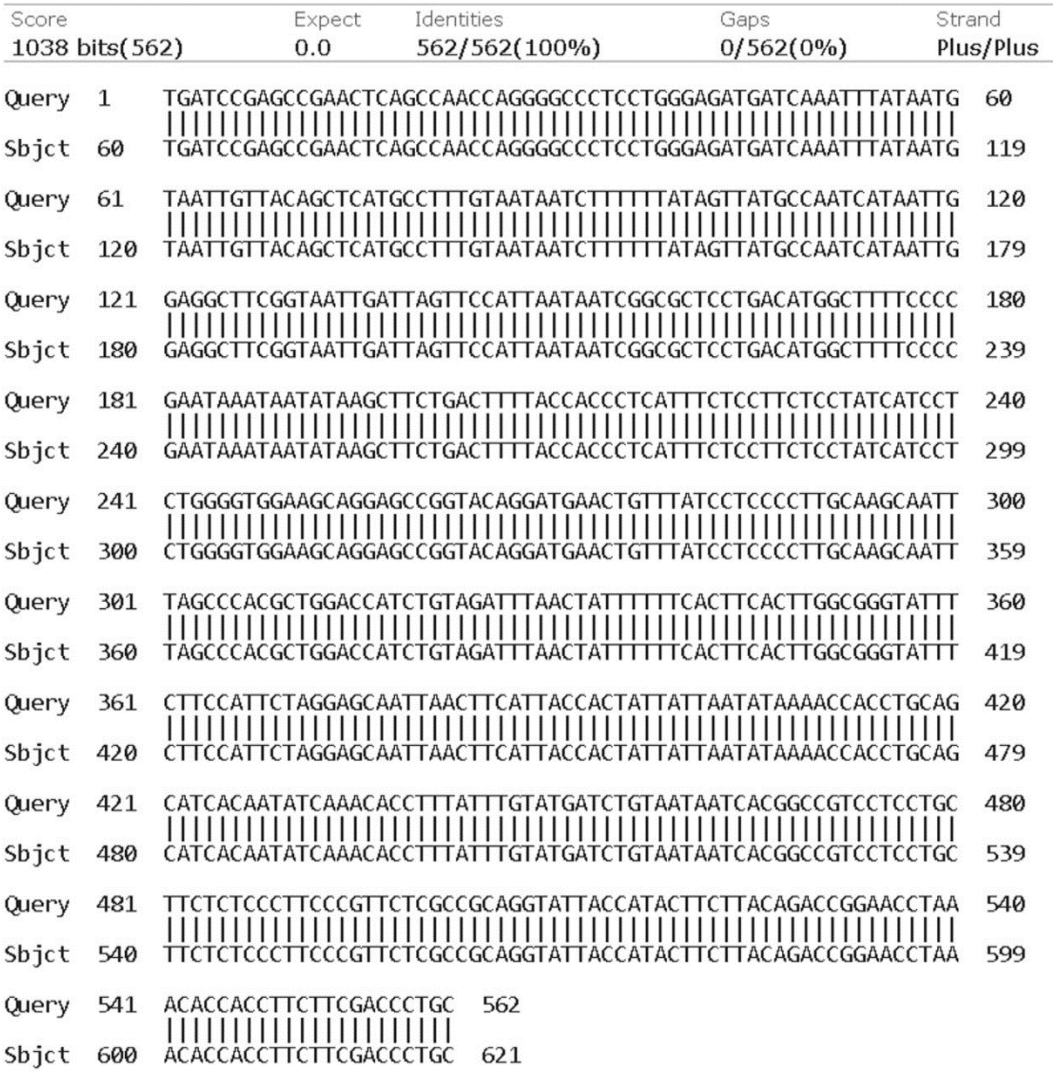

Figure 4. The sequences of nitrogen DNA base is identic between the sample of this study and other research samples

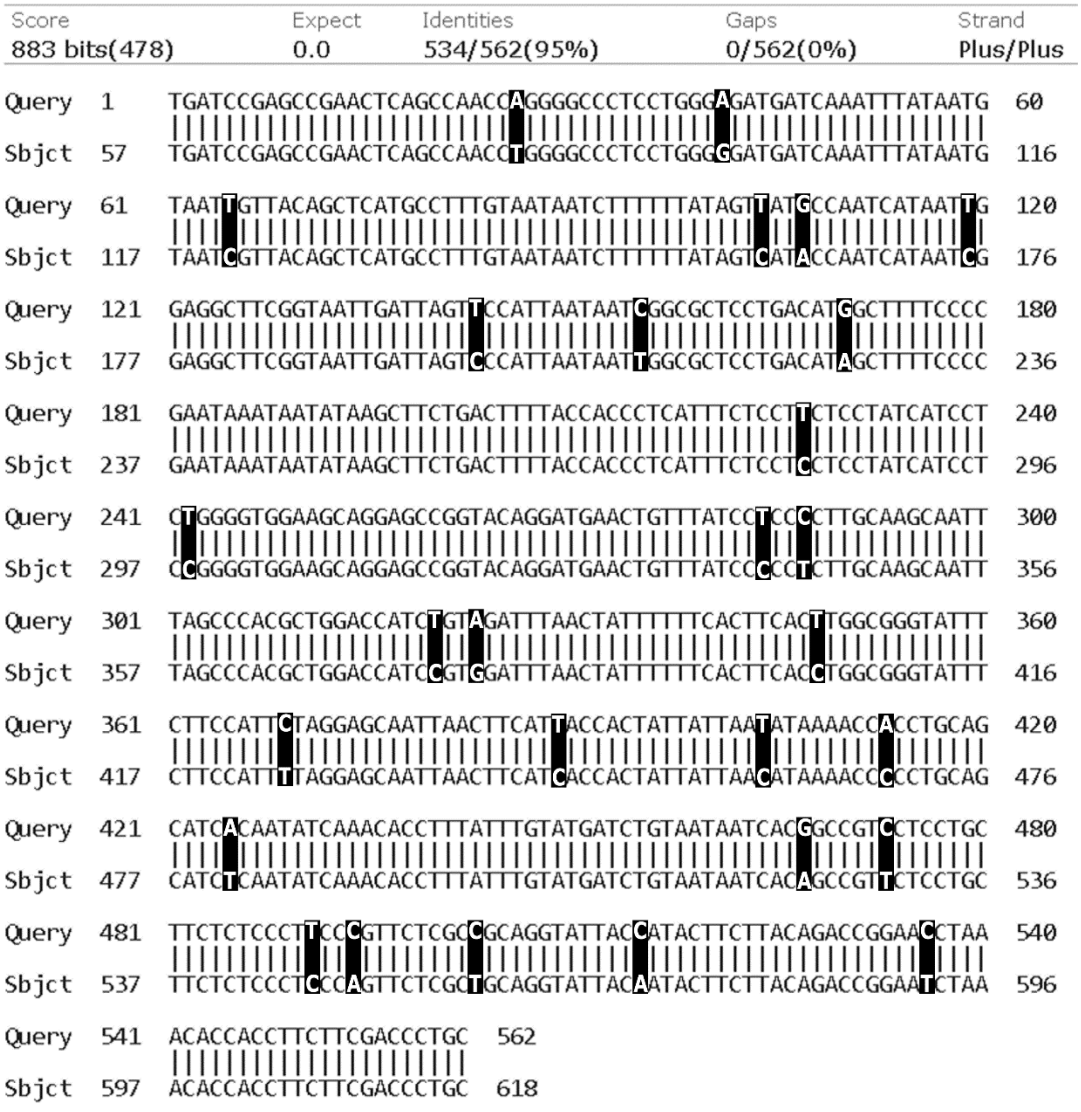

Figure 5. The different of DNA sequences between the sample of this study and other research samples

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,

CONCLUSIONS

There is a relationship between *P. reticulata* species in East Java from Surabaya, Jombang, Malang and Batu. They are ~~was~~ identical and are ~~had~~ in the same group in the phylogen~~y~~etic tree. *P. reticulata* from East Java ~~was~~ is also identical and ~~had a~~is in the same phylogenetic~~y~~ group with

species from other regions such as Sukabumi, West Java (KU692776.1); Pandeglang, Banten; Dominican Republic; and Myanmar even though they ~~were~~ are genetically different and ~~had~~ placed in different group ~~of~~ from *P. reticulata* from the South African; Brazil; and Sukabumi, West Java (KU692775.1).

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Authors sincerely thank to Mr. Setyanto, Mr. Suwarni, and Mr. Sunarto as a laboratory assistant and many other colleagues in Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya who kindly helped for operating laboratory instruments and in writing process of our manuscript.

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DNA Barcoding: A Study of Guppy Fish (*Poecilia reticulata*) in East Java, Indonesia ~~Based on Cytochrome c Oxidase Subunit I (CO~~

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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, r~~R~~esearch about *P. reticulate* is limited even though it is a well-known fish species in Indonesia. The purpose of study ~~is~~ was to identify ~~of~~ 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. ~~Research about *P. reticulate* is limited even though it is well-known fish species in 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 ~~phylogeny~~ 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~~There were two groups of *P. reticulata* for phylogeny tree. The first group was obtained through~~ that there were two groups of *P. reticulata*. The first group was obtain through species~~species samples from East Java~~ from East Java, ~~;~~ Sukabumi, West Java (KU692776.1),~~;~~ Dominican Republic,~~;~~ Pandeglang, Banten~~;~~ and Myanmar. The second group was~~. The second group was~~ *P. reticulata* from southern Africa,~~;~~ Brazil,~~;~~ and Sukabumi, West Java (KU692775.1).[M1] 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**RESULTS**[M2] ~~The~~ phylogeny ~~phylogenetic tree provides information about population~~ classification based on evolutionary relationships.
The~~se~~ findings of this study have important implication for the development ~~developing for~~of advance research about adaptation, phylogeny, and evolution of fish, especially of guppy fish.

Keywords: *Poecilia reticulata*, DNA barcoding, COI gene, phylogeny

INTRODUCTION

The guppy (*Poecilia reticulata*) is a freshwater fish and a member of the ~~family~~ 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 female~~s guppies~~. The ~~M~~males ~~guppies~~ 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 spec~~s~~ies of freshwater fish in the Java Island, Indonesia. Several spec~~s~~ies~~-~~ are endemic, but their ecosystem and biota are currently threatened (Hubert et al., 2015). In the Sunda area, the threatened biodiversity ~~threat~~ 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 ~~P~~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 [M3](Nuryanto et al., 2018).

~~The purpose of the present study is to identify *P. reticulata* through DNA barcoding using the cytochrome c oxidase subunit I (COI) gene. It is useful to determine the phylogenetic relationship between *P. reticulata* populations in East Java, particularly in the river.~~Molecular data is more widely used to make phylogenetic trees. It ~~due to~~ 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[M4]~~. It found~~ in the mitochondrial genome~~organelles~~ ~~and~~ has been generally applied to identification and research of animal biodiversity including fish[M5] (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* ~~research~~ 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 ~~is~~ was to identify *P. reticulata* through DNA barcoding using

the cytochrome c oxidase subunit I (COI) gene. It ~~is~~ was expected to be useful to determine the phylogenetic relationship between *P. reticulata* populations in East Java, particularly in the river. [U6]

~~**BENEFIT**~~ [M7]

METHODS

Study Area and Sampling

The ~~samples~~ sampling process were conducted from January to February 2018. The ~~F~~fish ~~was~~ 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 ~~was~~ were obtained with 2 fish from each sampling location. ~~It was performed to DNA analysis.~~ 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).

TABLE 1. Sampling locations

	Sample Code	Sampling Location (City/ Regency)	Coordinate	
1 2	A1 A2	Surabaya	7°16'36,1"LS	112°45'44,9"BT
3 4	B1 B2	Jombang	7°26'24,1"LS	112°17'45,5"BT
5 6	C1 C2	Malang	8°03'55,3"LS	112°37'48,4"BT
7 8	D1 D2	Batu	7°51'54,0"LS	112°31'45,1"BT

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 ~~isolation process~~ was ~~obtained~~ isolated from muscle tissue or meat of fish using Jena 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[M8] used for the next step, namely DNA amplification. If the isolated DNA sample is not used, it must be stored at -20^{o}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 perfo rmed to examine the DNA samples and the base ~~length~~ pairs (bp). The amplified target DNA ~~amplified~~ was from the ~~the base length pairs of~~ mitochondrial COI gene with a base length of ~~was~~ around 600 bp ~~(electro image)~~.[M9]

TABLE 2. PCR materials

	Material	Concentration	Volume (µL)
1	kit *KAPA2G Fast ReadyMix*	1x	24
2	Primer FishF1	0.5 [U10]Mm	2.5
3	Primer FishR1	0.5 Mm	2.5
4	ddH_2O	-	16
5	DNA sample	10-100 ng	2
6	Total	-	50

TABLE 3. PCR Condition

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

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 Gene bBank 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 GenBe bank. 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 of 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).

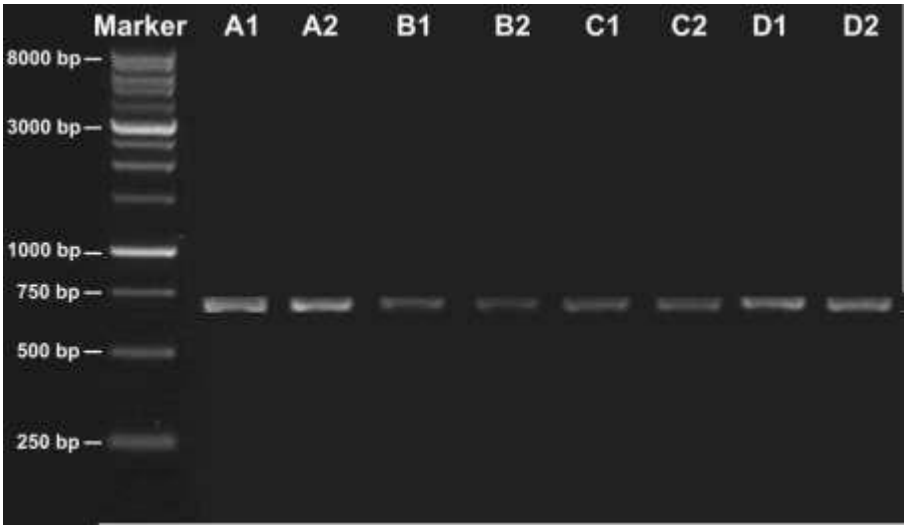

Figure 2. DNA electrophoresis result of COI gene

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), ~~suggested that~~ 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 ~~of~~ 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 ~~identification of~~ fish species (Chang et al., 2016).

In the present study, DNA sequences from *P. reticulata* in East Java and sample sequences from Gene ~~e~~ Bank'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*. ~~They were~~ used as out groups. [M11]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

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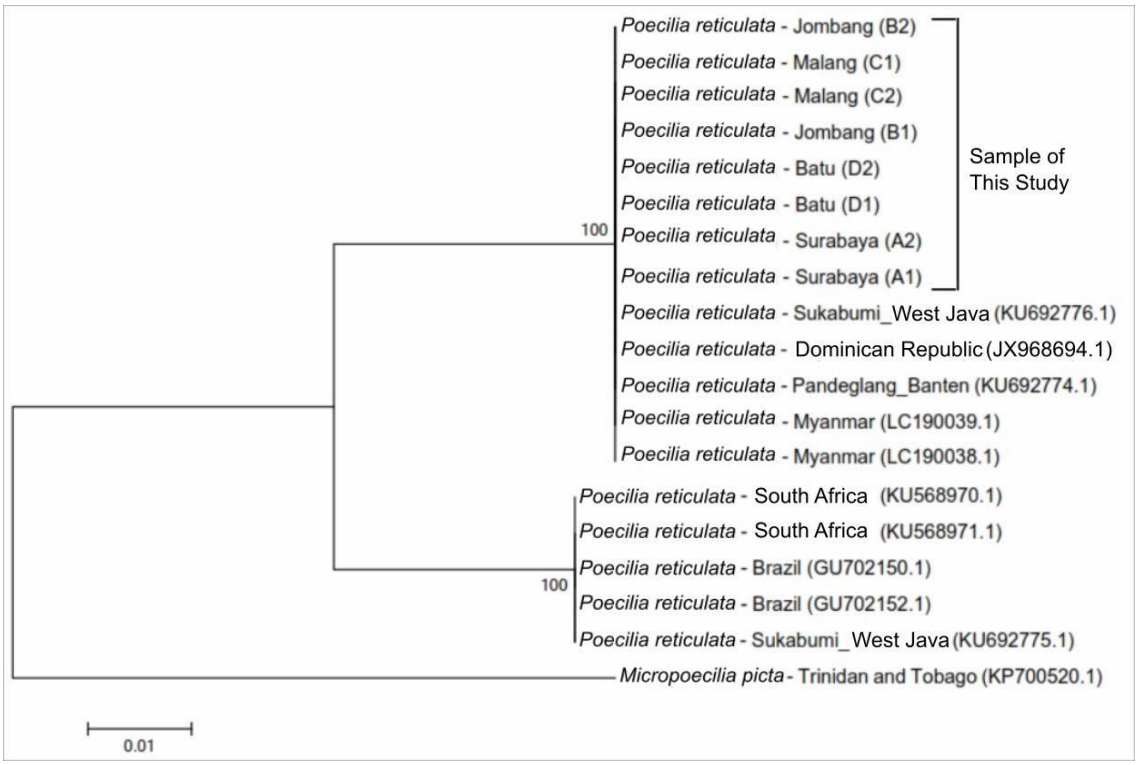

Figure 3. Phylogeny trees based on DNA sequences along with secondary data from Gene ~~b~~Bank (species name followed by origin area and sample code)

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 ~~namely~~ 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 ~~in~~ 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 [M13](Ellstrand & Schierenbeck, 2000), and disguise adverse mutations (Loewe & Hill, 2010). Tarallo et al. (2016) revealed that salinity and migration ~~can't~~ ~~impact~~ affect not only the~~on~~ physiological and morphological characters but also the genes character[M14] (nucleotide base consist of G and C) of teleost~~in fish (Tarallo et al., 2016)~~. 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~~s~~ is the relationship based on identical DNA or protein sequence composition to estimate the evolutionary process and evolutionary relationships of living things.

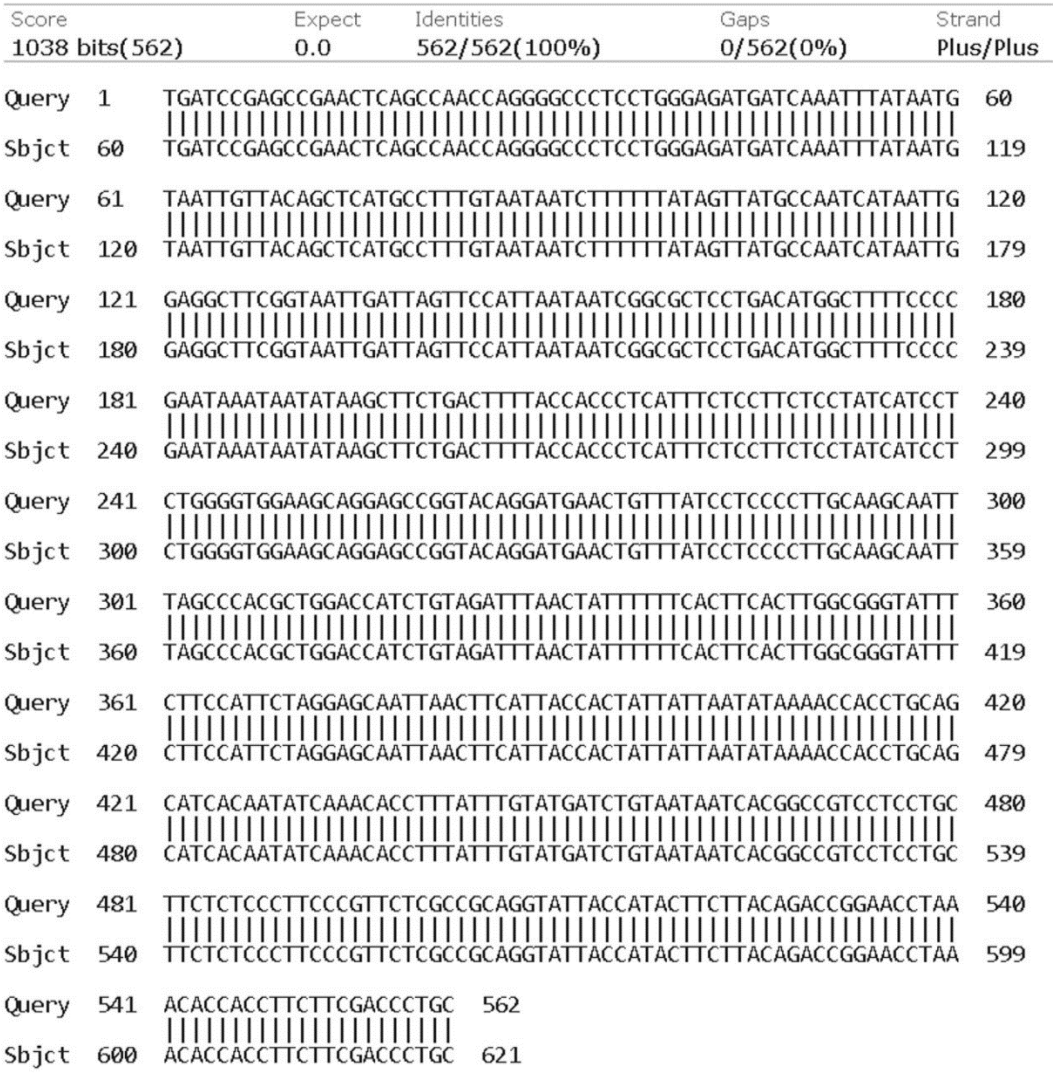

Figure 4. The sequences of nitrogen DNA base is identic between the sample of this study and other research samples

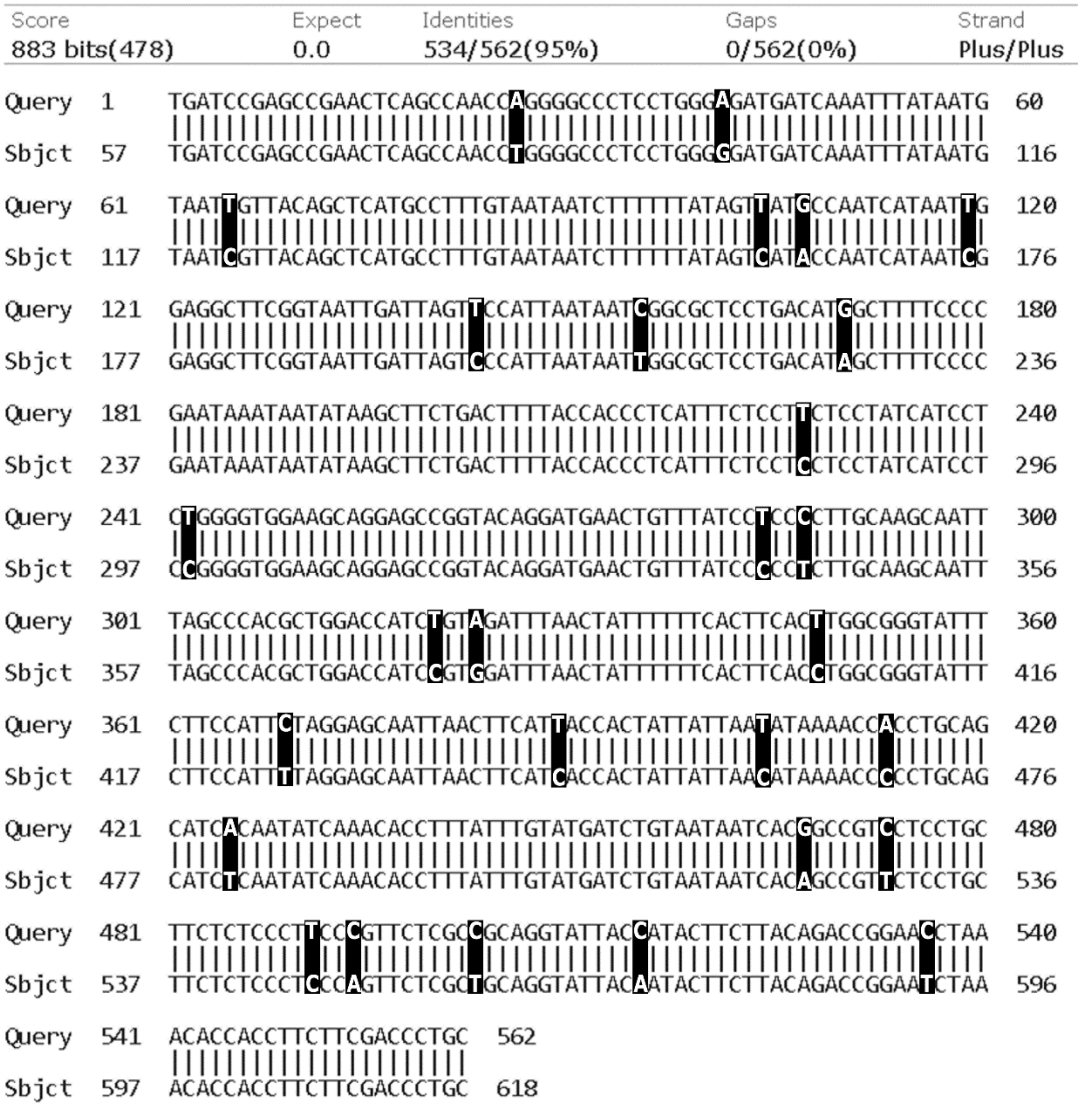

Figure 5. The different of DNA sequences between the sample of this study and other research samples

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,

CONCLUSIONS

There is a relationship between *P. reticulata* species in East Java from Surabaya, Jombang, Malang and Batu. They are ~~was~~ identical and are ~~had~~ in the same group in the phylogen~~y~~etic tree. *P. reticulata* from East Java ~~was~~ is also identical and ~~had a~~is in the same phylogenetic~~y~~ group with

species from other regions such as Sukabumi, West Java (KU692776.1); Pandeglang, Banten; Dominican Republic; and Myanmar even though they ~~were~~ are genetically different and ~~had~~ placed in different group ~~of~~ from *P. reticulata* from the South African; Brazil; and Sukabumi, West Java (KU692775.1).

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DNA Barcoding: A Study of Guppy Fish (*Poecilia reticulata*) in East Java, Indonesia ~~Based on Cytochrome c Oxidase Subunit I (CO~~

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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, r~~R~~esearch about *P. reticulate* is limited even though it is a well-known fish species in Indonesia. The purpose of study ~~is~~ was to identify ~~of~~ 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. ~~Research about *P. reticulate* is limited even though it is well-known fish species in 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 ~~phylogeny~~ 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~~There were two groups of *P. reticulata* for phylogeny tree. The first group was obtained through~~ that there were two groups of *P. reticulata*. The first group was obtain through species~~species samples from East Java~~ from East Java, ~~;~~ Sukabumi, West Java (KU692776.1),~~;~~ Dominican Republic,~~;~~ Pandeglang, Banten~~;~~ and Myanmar. The second group was~~. The second group was~~ *P. reticulata* from southern Africa,~~;~~ Brazil,~~;~~ and Sukabumi, West Java (KU692775.1).[M1] 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**RESULTS**[M2] ~~The~~ phylogeny ~~phylogenetic tree provides information about population~~ classification based on evolutionary relationships.
The~~se~~ findings of this study have important implication for the development ~~developing for~~of advance research about adaptation, phylogeny, and evolution of fish, especially of guppy fish.

Keywords: *Poecilia reticulata*, DNA barcoding, COI gene, phylogeny

INTRODUCTION

The guppy (*Poecilia reticulata*) is a freshwater fish and a member of the ~~family~~ 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 female~~s guppies~~. The ~~M~~males ~~guppies~~ 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 spec~~s~~ies of freshwater fish in the Java Island, Indonesia. Several spec~~s~~ies~~-~~ are endemic, but their ecosystem and biota are currently threatened (Hubert et al., 2015). In the Sunda area, the threatened biodiversity ~~threat~~ 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 ~~P~~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 [M3](Nuryanto et al., 2018).

~~The purpose of the present study is to identify *P. reticulata* through DNA barcoding using the cytochrome c oxidase subunit I (COI) gene. It is useful to determine the phylogenetic relationship between *P. reticulata* populations in East Java, particularly in the river.~~Molecular data is more widely used to make phylogenetic trees. It ~~due to~~ 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[M4]~~. It found~~ in the mitochondrial genome~~organelles~~ ~~and~~ has been generally applied to identification and research of animal biodiversity including fish[M5] (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* ~~research~~ 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 ~~is~~ was to identify *P. reticulata* through DNA barcoding using

the cytochrome c oxidase subunit I (COI) gene. It ~~is~~ was expected to be useful to determine the phylogenetic relationship between *P. reticulata* populations in East Java, particularly in the river. [U6]

~~**BENEFIT**~~ [M7]

METHODS

Study Area and Sampling

The ~~samples~~ sampling process were conducted from January to February 2018. The ~~F~~fish ~~was~~ 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 ~~was~~ were obtained with 2 fish from each sampling location. ~~It was performed to DNA analysis.~~ 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).

TABLE 1. Sampling locations

	Sample Code	Sampling Location (City/ Regency)	Coordinate	
1 2	A1 A2	Surabaya	7°16'36,1"LS	112°45'44,9"BT
3 4	B1 B2	Jombang	7°26'24,1"LS	112°17'45,5"BT
5 6	C1 C2	Malang	8°03'55,3"LS	112°37'48,4"BT
7 8	D1 D2	Batu	7°51'54,0"LS	112°31'45,1"BT

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 ~~isolation process~~ was ~~obtained~~ isolated from muscle tissue or meat of fish using Jena 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[M8] used for the next step, namely DNA amplification. If the isolated DNA sample is not used, it must be stored at -20^{o}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 perfo rmed to examine the DNA samples and the base ~~length~~ pairs (bp). The amplified target DNA ~~amplified~~ was from the ~~the base length pairs of~~ mitochondrial COI gene with a base length of ~~was~~ around 600 bp ~~(electro image)~~.[M9]

TABLE 2. PCR materials

	Material	Concentration	Volume (µL)
1	kit *KAPA2G Fast ReadyMix*	1x	24
2	Primer FishF1	0.5 [U10]Mm	2.5
3	Primer FishR1	0.5 Mm	2.5
4	ddH_2O	-	16
5	DNA sample	10-100 ng	2
6	Total	-	50

TABLE 3. PCR Condition

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

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 Gene bBank 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 GenBe bank. 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 of 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).

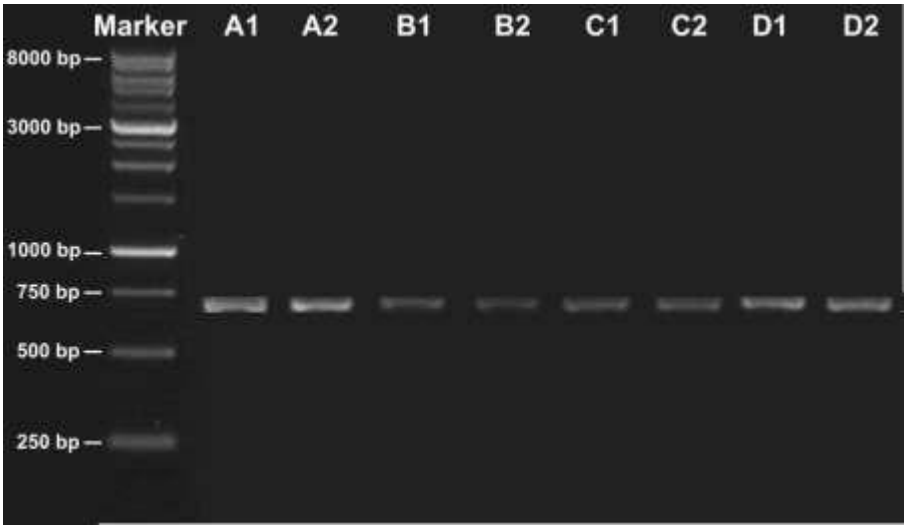

Figure 2. DNA electrophoresis result of COI gene

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), ~~suggested that~~ 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 ~~of~~ 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 ~~identification of~~ fish species (Chang et al., 2016).

In the present study, DNA sequences from *P. reticulata* in East Java and sample sequences from Gene ~~e~~ Bank'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*. ~~They were~~ used as out groups. [M11]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. ~~In the reconstruction of phylogenetic trees, molecular data is more widely used due to it is considered more stable in the evolutionary process compared to morphological data (Dharmayanti, 2011).~~[M12]

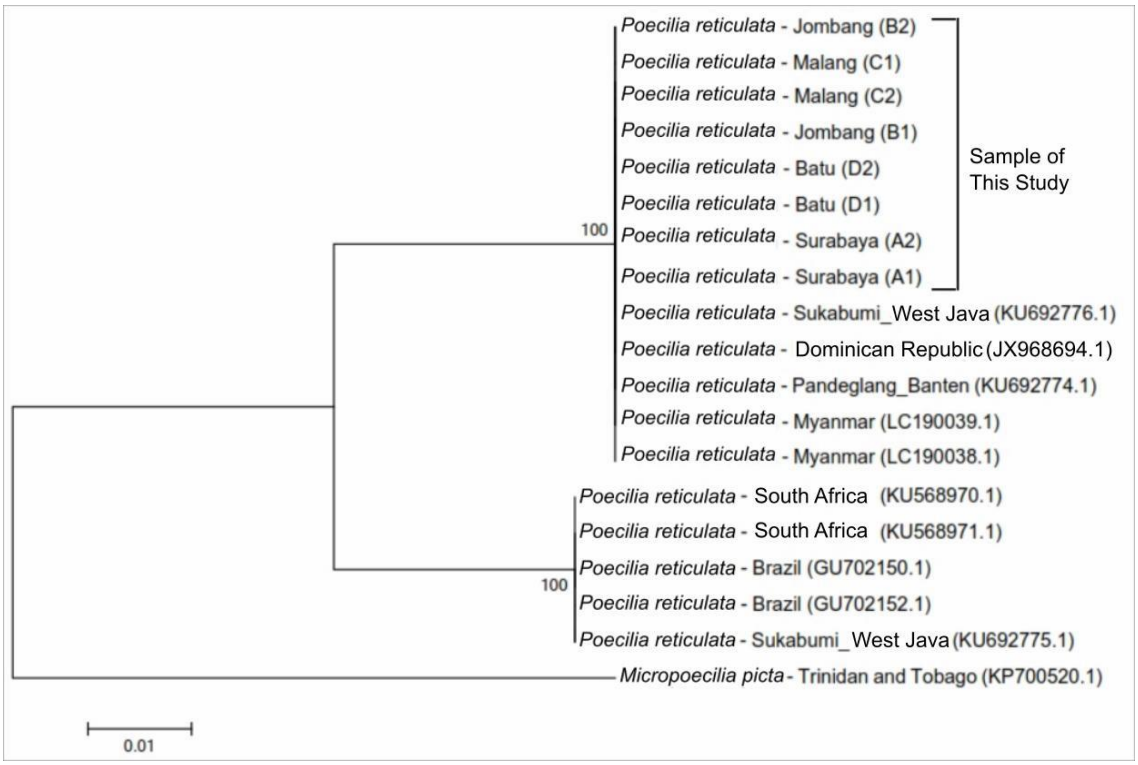

Figure 3. Phylogeny trees based on DNA sequences along with secondary data from Gene ~~b~~Bank (species name followed by origin area and sample code)

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 ~~namely~~ 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 ~~in~~ 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 [M13](Ellstrand & Schierenbeck, 2000), and disguise adverse mutations (Loewe & Hill, 2010). Tarallo et al. (2016) revealed that salinity and migration ~~can't~~ ~~impact~~ affect not only the~~on~~ physiological and morphological characters but also the genes character[M14] (nucleotide base consist of G and C) of teleost~~in fish (Tarallo et al., 2016)~~. 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~~s~~ is the relationship based on identical DNA or protein sequence composition to estimate the evolutionary process and evolutionary relationships of living things.

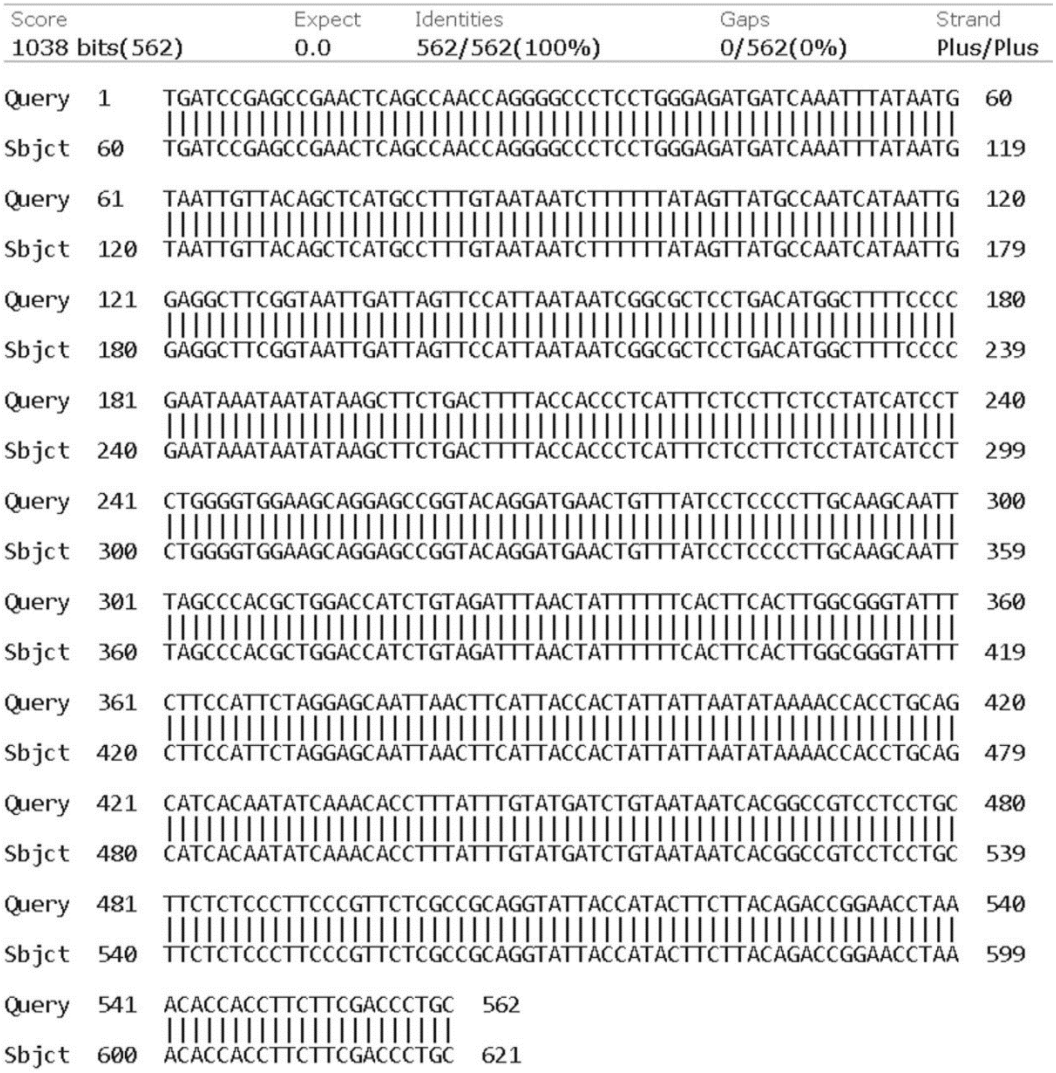

Figure 4. The sequences of nitrogen DNA base is identic between the sample of this study and other research samples

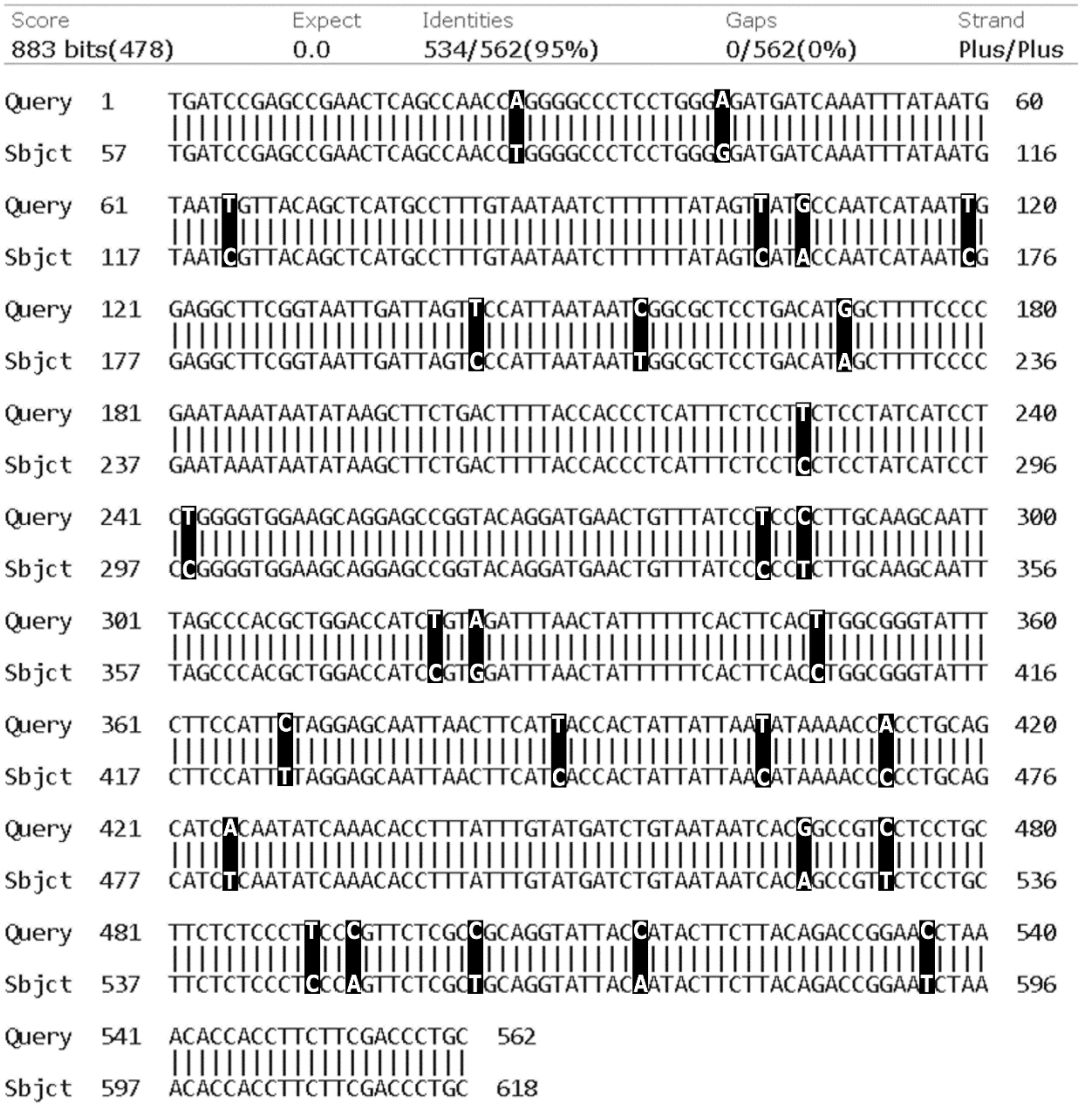

Figure 5. The different of DNA sequences between the sample of this study and other research samples

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,

CONCLUSIONS

There is a relationship between *P. reticulata* species in East Java from Surabaya, Jombang, Malang and Batu. They are ~~was~~ identical and are ~~had~~ in the same group in the phylogen~~y~~etic tree. *P. reticulata* from East Java ~~was~~ is also identical and ~~had a~~is in the same phylogenetic~~y~~ group with

species from other regions such as Sukabumi, West Java (KU692776.1); Pandeglang, Banten; Dominican Republic; and Myanmar even though they ~~were~~ are genetically different and ~~had~~ placed in different group ~~of~~ from *P. reticulata* from the South African; Brazil; and Sukabumi, West Java (KU692775.1).

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