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Cytochrome C Oxidase Subunit 1 Gene (COI) for Identification and Genetic Variation of Loaches (*Nemacheilus fasciatus*)

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KEYWORDS

Nemacheilus fasciatus; Cytochrome Oxidase C Subunit I (COI); Genetic variation.

Abstract *Nemacheilus fasciatus* is an Indonesian freshwater fish species that can be found in river waters on Java. This fish has a morphological similarity between species. This study aims to identify species of samples found and know their genetic kinship. This study used the Cytochrome Oxidase subunit I (COI) gene as a molecular marker, and then the results were analyzed using MEGA X software. The genetic structure and phylogeny of *N. fasciatus* sequences were combined with outgroup species from GenBank and analyzed using Maximum Likehood (ML), Pairwise Genetic Distance and Bootstrapping Phylogeny Model of Kimura 2 Parameters. The results showed that the primary pairs of LCO1490 and HCO2198 used to amplify the sample *N. fasciatus* with COI as a marker. The nucleotide frequencies of these loaches are A=26.5%, T=23.8%, C=19.9% and G=29.9%. The estimated Transition/Transversion bias (R) is 0.60. The number of haplotype diversity (Hd) was 0.972, and nucleotide (Pi) diversity was 0.05115. The kinship of *N. fasciatus* compared to the outgroup is closer to *N. pallidus* than *N. chrysolaimos*. Research needs to be conducted with a larger sample size for the genetic diversity of *N. fasciatus* in Indonesia.

Introduction

Genetic characteristics of *N. fasciatus* were previously studied (Valen et al., 2019). The loach fishes of the family Nemacheilidae has about 667 known species with 24,6% new description for the last ten years. Nemacheilid loaches of the genus Nemacheilus have more than 450 species distributed in the south of China, Southern Asia, Baluchistan, Western Irian, and East Africa (Kottelat *et al.,* 1993). *Nemacheilus fasciatus* is one species from this genus. In Indonesia, *Nemacheilus fasciatus* is spread in rivers on Java and is primarily found in Central Java and East Java. In the local area, these fish are used as consumption fish with a relatively high selling price. Some aquascape hobbyists use it for aquarium fish. In some rivers, these fish are increasingly challenging to find.

This loach has some particular characteristic of morphology. Dorsal fin shorter than other species in the same genus (7 or 8 branch fin), longitudinal lines form a band (dark blotch) throughout the body towards the caudal fin. *N. fasciatus* has large black eyeballs. Nostrils are close to each other, tubular but not extended as a whistle. Semicircular mouth, slightly fleshy lips, very wrinkled, upper lip with a pair of barbells. These fish have dark blotches of 14-18 from anterior to posterior and have 11-12 dorsal saddle patterns. These fish were spread in Java and Sumatra. Male has a slimmer body shape with brighter body colouring than females. The male tail is usually red, while the same is not found in females. Species in the family Nemacheilidae have a relatively high morphological similarity that many taxonomic problems are located in species placement

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(Hadiaty, 2014). This is due to the difficulty of identifying the family of Nemacheilidae based on morphological characteristics so that it requires taxonomic taxation through molecular validation to confirm species and know their genetic relationship.

Checking species and genetic relationships can be done using DNA barcoding. DNA barcoding provides speed and accuracy in species identification, focusing on analysis in small segments of mtDNA. DNA barcoding can be a solution to the current taxonomic and has been developed to identify species because it is relatively easy to do compared to other techniques. DNA barcoding can be practically applied and as a tool to support other studies by selecting one or several loci that can be routinely sequenced and can be relied upon to identify large numbers of samples and diverse and easy compared between species. In addition to identifying cryptic taxa (Jaafar *et al.,* 2012), barcoding DNA has applicative functions such as looking at genetic characteristics, checking genetic diversity and genetic relationship ecological survey, and confirming samples' identity and phylogenetic analysis and evolutionary rates (Tang *et al*., 2011).

One DNA barcoding compatible with use is Cytochrome C Oxidase subunit 1 (CO1) gene fragment molecular analysis-based species. The COI gene is a region that encodes proteins in a large number of copies in cells. This gene does not experience large variations in length, strong secondary structures, or repetitions of single nucleotides that are sufficiently dense (Hollingsworth *et al*., 2011). Identification of species with DNA techniques barcoding has been widely used. Cytochrome Oxidase gene Sub Unit I (COI) is a barcode DNA. They were commonly used as a reference in genetic identification. COI gene reported having the potential for a low mutation rate compared to the cytochrome b gene. The COI gene is also widely used in tree analysis phylogenetic, genetic diversity, history evolution, and population genetics (Hebert *et al.* 2003).

Application of DNA barcodes using the COI gene to identify species in Nemacheilidae has been successfully carried out by several researchers, including Patil et al. (2016) in India, Sayyadzadeh et al. (2016) in Iran, and Pandey (2016) in India. But research on the use of COI to identify and analyze the genetic diversity of *N. fasciatus* in Indonesia has not been published. In this research, some DNA sequences were obtained by isolating specimen samples, and some of them were downloaded from GeneBank. Then the result was analyzed using the MEGA X program. This study aimed to identify the loach species from Pasuruan and Blitar (East Java Province), based on CO1 gene fragment DNA sequence and their genetic variations. Genetic variations to be known in this study include comparing base composition, probability of nucleotide substitution with Maximum Likelihood (ML) analysis, haplotype, genetic distance, and phylogenetic construction. The former is expected to support data for loaches resources management in Indonesia and augment the GenBank data of loaches DNA sequences.

Materials and Methods

Method of collecting data

Samples in the form of fish fins are collected from Pasuruan Region (2 samples) and Blitar Region (3 samples) of East Java, Indonesia. Five samples were analyzed in this research and stored in ethanol 96%. Sampling is collected during the months of October to November 2018. Some comparative data is downloaded from GeneBank to be analyzed and compared. Then produce data in the form of Mitochondrial DNA sequences control of the Cytochrome Oxidase I (COI) region. The sequence of mitochondrial DNA is widely used to analyze phylogeny trees because it has a fast evolutionary rate.

DNA extraction, PCR, and sequencing

Extraction method using Qiagen KIT. Subsequently, < 25 mg of sample was taken and cut into small pieces and put into a 1.5 ml microcentrifuge tube. Then mix using a vortex, incubate 56°C for 10 minutes and separate using a centrifuge to get a supernatant.

DNA amplification or propagation is done by PCR (polymerase chain reaction) method. The extracted sample was amplified at the COI locus (Cytochrome Oxidase subunit I) with the Hotstart method. The parameters used in this method are as follows: denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds, and the PCR process is repeated as many as 38 cycles. In this method, two primers are used: the forward primary LCO1490 with nucleotide sequences as follows: 5'-ggtcaacaaatcataaagatattgg3' and primary back (reverse) HCO2198 with nucleotide sequences as follows 5'-taaacttcagggtgacca aaaaatca-3'.

Electrophoresis is done using 1% agarose gel and with colouring ethidium bromide (EtBr). 4 µL PCR products are mixed with 1 µL loading dye and put into gel wells. DNA low mass ladder is used as a marker (marker). Electrophoresis is run with 200-volt voltage, 400 mA current, for 15 minutes. DNA bands are seen using lights UV-translator.

DNA sequencing is the method for determining the nucleotide sequence contained in DNA. Reading sequence nucleotides were carried out by First Base sequencing services by using a sequencer machine automatic. The nucleotide sequences are in the form of AB1 files that analyze using 5.10.01-DnaSP software and MEGA X.

Data analysis

The sequence was aligned usingClustalW MEGA X software. The aligned sequences were then exported to the NCBI for species identity analysis using the Basic Local Alignment Search Tool (BLAST) program. The outcome of DNA matching to the first sequence number's highest homologous value was expressed as sample identity. Further identification was made using the Disparity Index Test of Substitution Pattern or intersequential disparity test. Monte Carlo (1,000 replications). The analysis employed version X-MEGA. The following steps were to measure each individual's genetic distance and build the phylogenetic tree to determine the species identity. Genetic distance differences between taxa were calculated based upon the p-distance model. The kindship between taxa was analyzed based on the maximum-likelihood (ML) method and reconstructed through 1,000 replications (*bootstrap*). Genetic diversity was analyzed based on CO1 sequence base composition variable using version X-MEGA software (Tamura *et al*., 2011), while the haplotype variables, haplotype diversity, and nucleotide diversity used version 5.10.01-DnaSP (Rozas *et al*. 2009).

Results and discussion

The amplification result using the PCR technique is an amplicon COI gene fragment from Nemacheilus fasciatus visualized using the electrophoresis method on the gel agarose 1%. Colouring of DNA molecules done using ethidium bromide (EtBr) gives an orange colour if seen with UV transilluminator.

The length of the sequence of nucleotide bases successfully amplified, which is 500-750 bp. The length of PCR products obtained good quality so that it can be continued at the purification and sequencing stage. Electrophoresis results are presented in Figure 1.

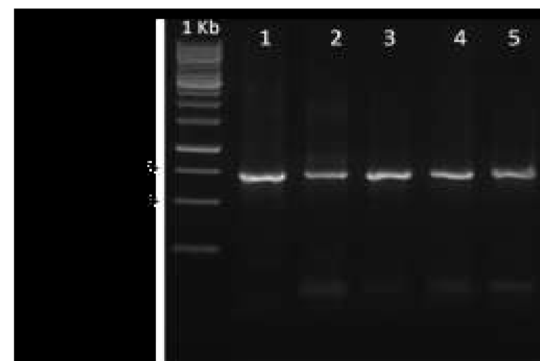

Figure 1. Electrophoresis result of *N. Fasciatus*

Electrophoresis results show that the five positive samples obtained an amplicon COI gene fragments are characterized by the existence of DNA bands in the gel well with the length between 500-700 base pairs (BP). This result ensures that the gene is amplified what is obtained is a gene target of the primer used. The nucleotide concentration of PCR products is determined based on the brightness of the band. Research DNA is compared with DNA bands ladder. Research results on well number 2 until number 7 show the brightness level, which approaches DNA ladder line 3 (80 ng). This means that the COI gene DNA concentration the amplification results have less concentration from 80 ng but more than 40 ng. This is appropriate with brightness on the DNA ladder tape. According to Invitrogen (2005), the first row DNA ladder tape has a concentration of 200 nanograms (ng), second to the sixth row respectively shows a concentration of 120 ng, 80 ng, 40 ng, 20 ng, and 10 ng.

All sequences of loaches nucleotide (682 bp) were successfully identified using BLAST. The five samples showed similarities above 97%, as shown in Table 1. Based on the BLAST analysis results, it can be concluded that the sequenced DNA samples have a high degree of similarity with the DNA sequences available at Genbank by above 90%, so it is confirmed as *Nemacheilus fasciatus* species.

Table 1. BLAST Results with GenBank Data.

Sample code	Species of BLAST outcome	Identity value (%)	Access code of NCBI
N._fasciatus_(Blitar_1)	*Nemacheilus fasciatus*	99.68%	KT960792.1
N._fasciatus_(Blitar_2)	*Nemacheilus fasciatus*	99.50%	KT960792.1
N._fasciatus_(Blitar_3)	*Nemacheilus fasciatus*	97.01%	KT960792.1
N._fasciatus_(Pasuruan_1)	*Nemacheilus fasciatus*	99.84%	KU692665.1
N._fasciatus_(Pasuruan_2)	*Nemacheilus fasciatus*	99.53%	KU692665.1

The length of the fragment for the sample loaches obtained has a long base (bp) that is longer than some of the other studies in different fishes but also found the base length (bp) is longer than the results of the research obtained. Differences in base length (BP) from the amplification results were caused due by differences in sample size, DNA quality found, primary specifics, primary length size, primary base composition, environment, food, and offspring (Shizuka and Lyon, 2008). But in different general differences in base length and primary use do not show any influence on the results of the analysis carried out.

Maximum Likelihood (ML) analysis results for the probability of nucleotide composition with 5 sequence of mitochondrial DNA COI regions of loaches *Nemacheilus fasciatus* obtained nucleotide frequencies are A=26.5%, T=23.8%, C=19.9%, and G=29.9% (Table 2). The estimated transition/transversion bias (R) is 0.60. Guanine ranks first as the most common base in the nucleotide sequence of *Nemacheilus fasciatus,* followed by adenine, thymine,, and cytosine. Guanine is a base with three hydrogen double bonds that are more difficult to separate,, so the chance of mutations in this base is smaller (Jusuf, 2001).

Table 2. Nucleotide Composition.

	T	C	A	G
Blitar 1	23.8	19.9	26.4	29.9
Blitar 2	23.7	19.9	26.4	30.0
Blitar 3	23.9	19.7	26.7	29.6
Pasuruan 1	23.8	20.0	26.3	29.9
Pasuruan 2	23.8	19.8	26.5	29.9
Avg.	23.8	19.9	26.5	29.9

The analysis of nucleotide composition at loaches showed that the average amount of guanine was found to be the highest. These results are closely related to the probability of nucleotide substitution, indicating that guanine substitution is higher (Table 3). In this study, an analysis of the Maximum Composite Likelihood Estimate was carried out to determine the pattern of substitution mutations. Substitution mutations are critical because the COI gene sequence's evolutionary process is based on nucleotide substitution with others during evolutionary time. Those substitution mutations consist of transitional substitutions and transversal substitutions. Transition substitution is a change between the purine base and between the pyrimidine bases, while the transverse substitution is the change that occurs between purine and pyrimidine (Puterbaugh and Burleugh, 2001).

Table 3. Probability of Nucleotide Substitution with Maximum Likelihood (ML) Analysis.

	A	T	C	G
A	-	*7.56*	*6.27*	**11.77**
T	*8.39*	-	**6.54**	*9.48*
C	*8.39*	**7.88**	-	*9.48*
G	**10.42**	*7.56*	*6.27*	-

Remarks: Each entry is the probability of substitution (r) from one base (row) to another base (column). Substitution patterns and rates were estimated under the Tamura and Nei (1993) model. Rates of different transitional substitutions are shown in bold, and those of transversions substitutions are shown in italics. Relative values of instantaneous r should be considered when evaluating them.

Bowater (2003) explains the transition substance the chance is higher than the substance of transversion. In general, it is assumed that the transition ratio to transverse is higher in animal genomes and mitochondrial DNA, probably as a result of the underlying chemistry because purines and pyrimidines have different conformational sizes, where purines have a bicyclic structure, and pyrimidine has a single ring structure, the transfer process may be more complicated than the transition process), and this is one reason why the transition frequency is higher than the transverse.

The highest value of substitution probability found at base G is base A, T, and C. It was closely related to the frequency of each nucleotide. The higher the nucleotide frequency is concerned, the greater the probability of substitution. The probability of transitional substitutions is higher than transversions substitutions. The transversionsal substitutions between purin with purin and between pirimidin with pirimidin are easier than transversions substitutions between purin with pyrimidines. The similarity of the molecule structure influences it. GeneBank sequences have a strong relationship that shown by The estimated Transition/Transversion bias (R) is 0.60. The five samples do not have a sequence of the same nucleotide. There are several different nucleotides (polymorphisms) that result from a point mutation. Eight polymorphisms of this sample are presented in Table 4.

Table 4. Nucleotide Mutation Position.

ID Samples	Nucleotide Position							
	14*	89*	90*	91*	126*	139*	166*	677**
Blitar 1	C	T	G	T	G	C	G	A
Blitar 2	C	G	T	G	G	C	G	A
Blitar 3	C	T	G	T	A	T	A	A
Pasuruan 1	C	T	G	T	G	C	G	C
Pasuruan 2	A	T	G	T	G	C	G	C

Basically, mutations can occur naturally and occur due to external factors. Natural mutations occur because of an error copying of DNA molecules when they occur in DNA replication. In contrast, mutations due to external factors occur due to radiation or the present exposure to chemicals. Even though it is in the body, there is a DNA repair system, mutation events still happening, and sometimes it still accumulates and carries over to the next generation. These mutations will result from the existence of evolution (Toha, 2011). Transition mutations are more frequent and easily occurs compared to transversion mutations. Research result shows that the eighth mutation is a transition mutation, which is between nucleotides adenine and guanine and nucleotides cytosine and thymine. This is caused by the possibility of a pairing error DNA molecule. The possibility of an error the couple allowed by Watson and Crick's concept is a couple's fault between purine and pyrimidine bases. Next, the research adds information that errors paired between purine and purine still may occur, while a partner's fault between pyrimidine and pyrimidine bases is not possible. That is why transition mutations are easier and often happen rather than transversion (Graur and Li, 2000).

The five samples have no order of the same nucleotide. There are several different nucleotides (polymorphisms) that result in there is a point mutation. Sixth polymorphism This sample is presented in Table 4. Substitution patterns and rates were estimated under the Kimura (1980) 2-parameter model, Maximum Likelihood Estimate of Substitution Matrix. This nucleotide consists of 500 bp invariable sites (monomorphic) DNA and 110 bp of variable sites (polymorphic) DNA. Alignment of the nucleotide sequence of the COI gene Nemacheilus fasciatus produces value singleton variables sites was 86 bp, and parsimony-informative sites were 25 bp.

Value shows variations in nucleotide bases in the form of distinguishing characteristics between species. The total number of mutations was 121 bp. The number of haplotype diversity (Hd) was 0.972, and nucleotide (Pi) diversity was 0.05115. A sequence of haplotypes is stated differently from other sequences, if they have a minimum of one different nucleotide. Based on polymorphism in Table 4, all five samples can be grouped into five haplotypes different. There is a haplotype diversity value category that is > 0 and > 0.5 included in the low category, while > 0.5 and < 1 are included in the high category (Hobbs et al., 2013). Nei (1987) classifies the ratio of the 0.1 - 4.4 haplotype as low category, a 0.5 - 0.7 medium category, and a 0.8 - 2.00 high category. Based on this category, the haplotype diversity of Nemacheilus fasciatus in the Pasuruan area and Blitar area is in the high category. The high genetic diversity was good, because low genetic diversity will be resulting in the emergence of negative traits, including decreasing growth, diversity in size, development of stability of organs, survival rate, and adaptation to environmental changes (Leary *et al.,* 1985).

Nei (1981) that the value of one species' genetic diversity depends on the size of the sample found. The overall haplotype diversity of mtDNA for some fish is in the range of 0.473-0.998. Barton (2010) explained that differences in the value of haplotypes between and within populations resulted from substitution, genetic insertion, or deletion.

In general, the source of genetic variation is caused by random marriage, enormous population size, migration, mutation, recombination, and natural selection (Barton, 2010). With the high genetic diversity, Nemacheilus fasciatus is thought to be caused by two factors: first is a large population that allows interbreeding among individual members; thus, each individual can meet other individuals, both with genotypes same or different from him. Cross-breeding like this helps increase the frequency of alleles one generation and is handed down to the next generation.

Genetic distance is used to see the proximity of genetic relations between individuals. The genetic distance of CO1 gene fragment to three species is displayed in the form of a matrix. Result of genetic distance using Pairwise Analysis Kimura 2 Parameter found overall mean distance is 0.295, with closest genetic distance is 0.0% and farthest 1.44%. It means from 631 bp only maximum 2 different nucleotides and tends low genetic variance. Sequence result from 5 samples obtains nucleotide with 689 bp. Most sequences are guanine nucleotides (G). The nucleotide composition for each sample can be seen in Table 5.

Table 5. Genetic Distance.

Species	1	2	3	4	5	6	7	8	9
N. fasciatus (Blitar 1)									
N. fasciatus (Blitar 2)	0.000								
N. fasciatus (Blitar 3)	0.007	0.007							
N. fasciatus (Pasuruan 1)	0.002	0.002	0.008						
N. fasciatus (Pasuruan 2)	0.003	0.003	0.010	0.005					
N. fasciatus (Jepara)	0.010	0.010	0.010	0.012	0.013				
N. fasciatus (Purwokerto)	0.003	0.003	0.003	0.005	0.007	0.007			
MF289074 N. palidus (Thailand)	0.088	0.088	0.091	0.088	0.092	0.088	0.088		
KU692664 N. chrysolaimos (Sukabumi)	0.162	0.162	0.162	0.162	0.167	0.156	0.162	0.158	

Genetic distance is used to form a phylogeny tree, then from this tree can be known between interspecies relationships. The phylogeny tree is constructed based on the p-distance method. Results of phylogeny analysis obtained a real separation between Indonesian species with species outgroups.

Each sample is compared with the whole Gene Bank data and selected one sequence with the highest resemblance. Every high-similar sequence are used in making phylogenetic trees. Results BLAST of the six samples is presented in Table 5. The five samples refer to the species Nemacheilus fasciatus. This is proven with BLAST results (Basic local alignment search tools) to Gene Bank data that shows that this sequence bears a resemblance high with fish Nemacheilus fasciatus. The results of the further analysis with trees phylogenetic are shown in Figure 2. The phylogenetic tree shows that the five samples are in one clade. Among species of Nemacheilus fasciatus, species from Jepara have the most distant kinship, while the sample species Nemacheilus fasciatus from Blitar 3 is closer to the species from Purwokerto downloaded from GenBank. As a comparison, sequences of *Nemacheilus palidus* and Nemacheilus Crhysolaimos species were also downloaded from GenBank.

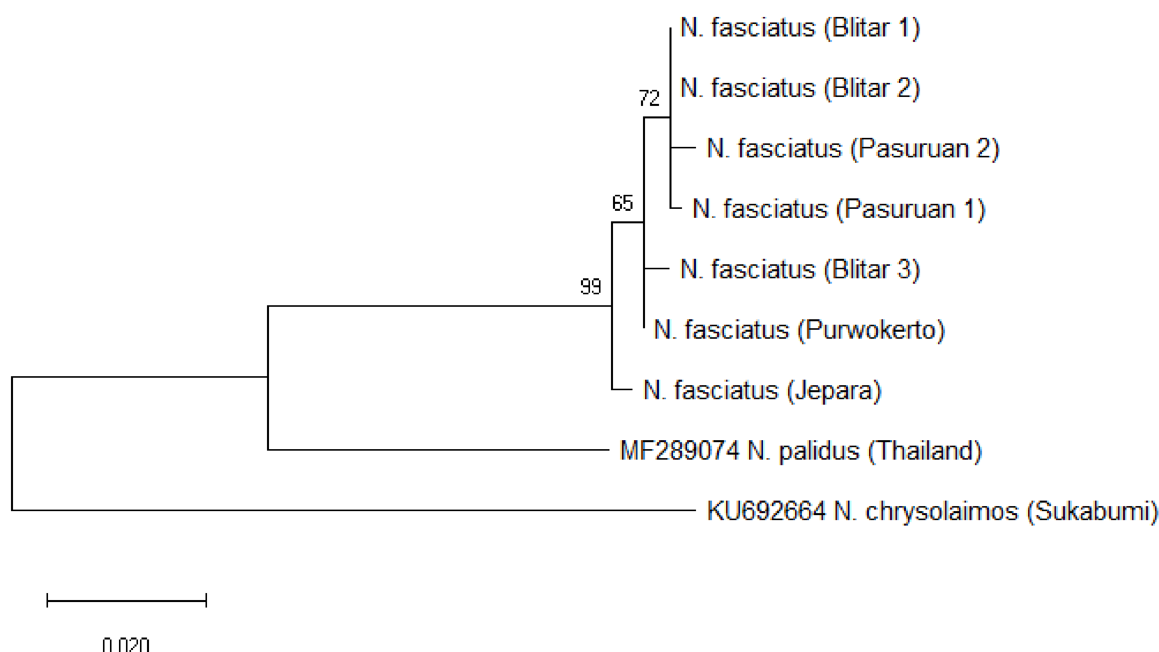

Figure 2. Molecular Phylogenetic Analysis by Maximum Likelihood method.

The five samples' genetic distance analysis compared to genebank data presented in Table 6 - the genetic distance between the five samples ranging from 0 to 0.167. The distance the genetic distance is best shown between outgroups, other research samples, and Genebank data with a range of 0.156 to 0.167. This genetic distance shows the level of the kinship of each sample and Genebank data. Following the phylogenetic tree, it appears that *Nemacheilus chrysolaimos* is the furthest relative compared to other species in one genus.

Populations with high genetic diversity have a better chance of life; this is because each gene has different responses to environmental conditions. The presence of various genes in individuals in the population provides an

opportunity to resist various existing ecological changes such as pollution (Islamy et al., 2017; Islamy 2019; Isroni et al., 2019; Kilawati and Islamy 2019; Islamy and Hasan 2020; Pardamean et al., 2021). High genetic diversity in fish populations can protect from various ecological disturbances. The process of transferring genetic material between populations of different locations influences genetic diversity (Hartl and Jones, 1998).

The results obtained indicate that the two fish populations are one offspring and migrate with the migration pattern simultaneously, resulting in these two populations becoming genetically similar. It also explains that although each population group is separated from one another, these two populations have genetic proximity and one common ancestor.

Molecular identification of research has shown taxonomic certainty (certainty taxonomy). It can be made as a reference for population genetic studies, stock assessment until sufficient data is reached to take action on resource management loaches through conservation. The wide variety of genetic diversity and specific haplotypes illustrates that there has not been a change in genetic structure in population loaches in Pasuruan and Blitar because they still have diverse genes. However, it is known that resource loaches are species that are used as target fishing for local consumption. However, fishing activities cannot be allowed to continue to take care of because they can affect the population structure, resulting in a decrease in a species' genetic diversity. This supports the view that the need for a strategy to protect biodiversity is needed through genetic conservation because biodiversity covers all aspects, including habitat diversity, community, population, and species. This genetic difference is considered important compared to species and ecosystems; this is because genetic resources are an important key for a species to survive until the next generation. The biodiversity or biodiversity crisis starts from the decreasing level of genetic diversity of a species.

Further research, we suggest to applicate this method to other native or non-native fish that appear in Indonesian waters, such as Arapaima (Fadjar et al., 2019), Aligator gar (Hasan et al., 2020), Channidae (Pratama et al., 2020), Gobiidae (Hasan et al., 2021), Cichlidae (Insani et al., 2020), and Cyprinidae (Hasan et al., 2019).

Conclusions and suggestion

The primary pairs were able to amplify the sample Nemacheilus fasciatus with the COI gene as markers. Nucleotide frequencies of these loaches are A=26.5%, T=23.8%, C=19.9% and G=29.9%. The kinship of 5 sequences of Nemacheilus fasciatus is close and when compared to the outgroup closer to N. pallidus than N. chrysolaimos. Research needs to be conducted with a larger sample size to determine the genetic diversity of N. fasciatus in Indonesia.

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