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Genetic Identification of Endemic Fish *Nomorhamphus liemi* Vogt, 1978 from Sigi, Central Sulawesi, Indonesia

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ABSTRACT: Sulawesi Island is geographically included in the Wallacea region, a transitional area between the Oriental and Australian zones, which makes Sulawesi's fresh waters have a high level of fish species diversity. Some fish species found in Sulawesi freshwater are endemic. Research on Sulawesi endemic fishes in rivers is not as much as research on endemic fishes in lakes. *Nomorhamphus liemi* or locally known as angculung is one of Sulawesi's endemic fishes. This species belongs to the sub-family *Zenarchopterinae*. The majority of species from the *Zenarchopterinae* sub-family are sexually dimorphic. This study aimed to molecularly identify angculung fish species using COI markers in the region. A total of nine angculung fish were collected and preserved using 96% alcohol. In the laboratory, tissue taken from the back of the dorsal fin was isolated and extracted, then amplified by PCR before sequencing. Sequencing results were validated on BLASTn, showing that nine samples belonged to *Nomorhamphus liemi* with 98-99% sequence similarity. The nucleotide base sequences were then analyzed using MEGA 7.0 software. The genetic distance of mackerel in these waters showed a close relationship. Phylogeny tree results showed population mixing so that mackerel in these waters can be managed in one management unit, especially in Sulawesi.

KEYWORDS: Sulawesi, COI gene, conservation, endemic fish, molecular identification

INTRODUCTION

Sulawesi Island is geographically included in the Wallacea region, a transitional area between the Oriental and Australian zones, which makes Sulawesi's fresh waters have a high level of fish species diversity. Some fish species found in Sulawesi freshwater are endemic (Peloso et al., 2015) [1]. There are 68 species from 7 families and 4 orders of freshwater endemic fish in Sulawesi. A total of 54 species were found in lake waters and 13 species were found in river waters. However, there are 11 endemic fish species that can be found both in rivers and lakes (Viswambharan et al., 2015) [2]. Recently, 6 (six) new species were found that are classified as endemic to Sulawesi. *Oryzias dopingdopingensis* was found in the Doping doping River. *Nomorhamphus versicolor* was found in Lake Lindu in 2019 (Hutama et al., 2017) [3]. *N. aenigma* found in Cerekang River in 2020 (Meng et al., 2018) [4]. *Schismatogobius limmoni* was found in Wera Nature Park, Palu, Central Sulawesi by Kraemer et al. (2019) [5]. *Oryzias landangiensis* was found in Cerekang River in 2022. *O. kalimpaaensis* was found in Lake Kalimpa in 2022 (Barman et al., 2018) [6]. Based on the above discoveries, the number of Sulawesi endemic fish species until 2022 is 74 endemic fish species and will likely continue to grow.

The majority of endemic fishes that have been recorded are freshwater fishes found in lakes. Research on Sulawesi endemic fishes in rivers is not as much as research on endemic fishes in lakes (Hutama et al., 2017) [7]. Daharuddin et al. (2016) [8] found several endemic fish species in Maros watershed rivers. These endemic fish species include beseng (*Marostherina ladigesi*), angculung (*N. liemi*), medaka (*O. celebensis*) and pirik (*Lagusia micracanthus*). These endemic fishes have economic value as ornamental fish, except for the pyrrhic fish, which is utilized as food fish.

N. liemi or locally known as angculung is one of the endemic fishes of Sulawesi (Kumar et al., 2016) [9]. This species belongs to the sub-family *Zenarchopterinae*. The majority of species from the *Zenarchopterinae* sub-family are sexually dimorphic. Sexually dimorphic means that there are differences in the shape of male and female fish in one species, ranging from size, color, structure, and morphology (Marzouk et al., 2016) [10]. Of the six genera of the *Zenarchopterinae* group, *Dermogenys, Hemirhamphodon,* and *Nomorhamphus* reproduce by giving birth, and the anal fin in males is modified into an andropodium. The andropodium has the function of delivering sperm to the female urogenital tract (Hutama et al., 2017) [11]. The presence of the andropodium suggests that fertilization of *N. liemi* is internal (Guimaraes-Costa et al. 2016) [12].

The unique body shape of *Zenarchopterinae* is its mouth shaped like a bird's beak, and its body color varies. This uniqueness is one of the reasons this species is used as an ornamental fish. Generally, the fish used as ornamental fish are *D. pusilla, N. liemi,* and *H.*

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pogonognathus. The sale of *Zenarchopterinae* fish as ornamental fish still relies on the results of capture in nature without paying attention to the status and condition of fish stocks in the waters. One solution to this problem is to make cultivation efforts. However, until now there have been no efforts to cultivate *D. pusilla, N. liemi,* and *H. pogonognathus*. In addition to continuous fishing activities, other threats to the sustainability of angculung fish in their habitat are habitat degradation, siltation of waters, introduction of foreign species, organic waste pollution, logging, land clearing and conversion for plantations, agriculture, and urbanization (Bingpeng et al., 2018) [13]. These anthropogenic activities can threaten the sustainability of angculung fish in their habitat. Therefore, angculung conservation efforts need to be carried out immediately. Basic information on the reproductive aspects of angculung is essential to support conservation efforts.

One common mitochondrial DNA marker used to distinguish taxonomic units is the barcode of the Cytochrome Oxidase subunit I (COI) gene. Even at the species level, phylogenetic relationships can be identified and analyzed using the COI gene. Additionally, morphological analysis and DNA barcoding are closely related, making DNA barcoding a good fit for classifying and confirming species identification. The COI gene is a suitable standardized barcode region that can be used to identify genetic relationships at the species level. Based on DNA Barcode COI, this work offers the first molecular scientific verification of the molecular identity of Nomorhamphus liemi, which was discovered in Sigi, Central Sulawesi, Indonesia. In order to preserve the sustainability of local species, it is envisaged that the molecular genetic information of brachiopods would be useful for future genetic population research and conservation initiatives. In the meantime, the knowledge of genetic diversity helps to increase population size and prevent inbreeding, both of which lead to the promotion of genetic diversity.

MATERIALS AND METHODS

Study area

This research was conducted from May to July 2023. Sampling was conducted in Sigi District, Central Sulawesi Province, Indonesia (Figure 1).

Figure 1. Sampling location of cangculung (*Nomorhamphus liemi*) in Sigi, Central Sulawesi, Indonesia

Procedures

Sampling

Five fish samples were used from each location. Samples for molecular analysis were taken from fish tissue at the bottom of the dorsal fin, put into a 1.5 mL microtube containing 96% alcohol. This process is done so that the samples used in the next process are not damaged. After that, the samples were brought to the laboratory for molecular analysis.

Sample preparation

Preparation was done to remove the alcohol content of the preserved samples. Fish samples were transferred into a new microtube and then washed using distilled water. The sample was vortexed for 1.5 minutes for five replicates. The sample was then dried and weighed as much as 0.03 grams. The weighed sample was then put into a new microtube for further DNA isolation and extraction.

DNA extraction

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This step was carried out using a commercial kit (Gene Aid) following the manufacturer's protocol with some modifications (Serdiati et al. 2020). Prepared samples were added with 200 µL of GT Buffer and then crushed using a pestle. The crushed sample was added 20 µL Proteinase K and shaken using a vortex for 30 seconds. After that, the sample was incubated at 60°C for 30 minutes with invert every 5 minutes. The sample was then added 200 µL GBT buffer and incubated again for 20 minutes with every 5 minutes inverted. After the incubation was complete, 200 µL of absolute ethanol was added to the sample and kept in the freezer for 30 minutes. The supernatant formed was transferred to a spin GD column and then centrifuged at 14000 G for 2 minutes. The bottom liquid from the centrifugation was discarded. The sample was added 400 µL of W1 buffer and centrifuged again at 14000 G for 30 seconds. The bottom liquid from the centrifugation was discarded. The sample was added 600 µL of W2 buffer and re-centrifuged at 14000 G for 30 seconds and the lower liquid was discarded. Centrifuged again at 14000 G for 3 minutes without adding solution. The spin GD column was transferred to a new microtube after centrifugation was complete. Elution was then performed twice. Elution was done by adding 50 µL of elution buffer (EB) which was previously heated at 60ºC. The sample was allowed to stand for 10 minutes at room temperature and then centrifuged again at 14000 G for 30 seconds.

DNA quality test

DNA quality testing was carried out using the electrophoresis method. This method uses 1.2% agarose gel soaked in 1x TAE buffer solution. Fluorescent is added as a nucleic acid dye (DNA or RNA) and loading dye is added as a DNA ballast in the agarose gel. The agarose gel was then observed under UV light (Zhang and Hanner 2019) [14].

Amplification and visualization of COI gene DNA fragments

Total DNA isolated and extracted was used as a DNA template for DNA amplification. The amplification process was carried out by PCR (Polymerase Chain Reaction) technique using My Taq HS Red Mix commercial kit. The amplified DNA fragment was the COI gene. The primers used were universal primers for several aquatic biota. Amplification was carried out in several stages, namely predenaturation (temperature 94°C for 5 minutes), denaturation (temperature 94°C for 45 seconds), annealing (temperature 54°C for 1 minute), elongation (temperature 72°C for 1 minute), postelongation (temperature 72°C for 5 minutes). Furthermore, PCR products were visualized using a 1.2% agarose gel on an ultraviolet machine.

DNA sequencing

DNA sequencing is the determination of the sequence of nucleotide bases contained in DNA. Sequencing is performed using the Sanger method. PCR products with good quality are sent to a sequencing service company for determination of nucleotide base sequences.

Data analysis

Validation of the COI gene nucleotide base sequence

The nucleotide base sequence obtained from the sequencing process was then validated. Validation was carried out by uploading the nucleotide sequence results to the Basic Local Alignment Search Tool-nucleotide (BLASTn) found on the NCBI website (www.ncbi.nlm.nih.gov). The results obtained are in the form of a percentage value of similarity with the species contained in the GenBank database. The similarity percentage value is used as a reference in validating the species.

Alignment of COI gene nucleotide sequences

The nucleotide base sequences obtained from the sequencing process were then aligned using MEGA 7 software. The nucleotide sequence of the mackerel COI gene with forward and reverse primers was edited and analyzed to obtain the DNA sequence of the COI gene. In addition to forward and reverse, nucleotide sequences were also aligned with outgroup species obtained from GenBank. The outgroup used is a species that is still in the same class and family as mackerel. The results of nucleotide sequence alignment are in the form of base length, nucleotide base composition, and mutation sites.

Genetic distance calculation

The genetic distance of COI nucleotide base sequences between Rastrelliger spp. species was analyzed using MEGA 7 software (Kumar et al. 2016) [9]. The results of genetic distance are in the form of a data matrix and can be used to analyze kinship relationships between species. Genetic distance of mackerel was also compared with outgroup species. A genetic distance of ≥ 3% indicates a difference in the species being compared.

Phylogeny analysis

Phylogeny analysis was conducted using MEGA 7 software (Kumar et al. 2016) [9]. Genetic distance matrix data were used for phylogeny tree reconstruction. Phylogeny tree construction using outgroup species was done for comparison. Phylogeny tree shows a relationship based on DNA or protein base sequence composition.

RESULTS AND DISCUSSION

Total DNA

The results of isolation and extraction of DNA from nine samples of *Nomorhamphus liemi* fish showed good DNA electrophoresis results because there were no smears (Figure 2). Good quality DNA will be in the form of bright and clear bands. DNA bands that look smeared indicate that there is still contamination from other components such as RNA and protein in the sample.

Amplification and visualization of COI gene DNA fragments

Amplification of COI gene DNA fragments by PCR technique was performed at an optimal annealing temperature (primer attachment) of 54°C. The length of nucleotide bases obtained was between 500-700 bp (Figure 2).

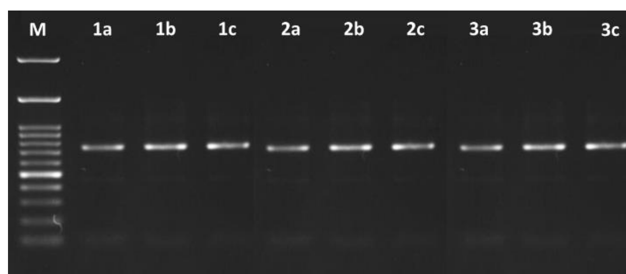

Figure 2. DNA band from PCR product testing

Validation of COI gene nucleotide base sequences

The COI gene nucleotide base sequences of the nine samples were uploaded to BLASTn (Basic Local Allignment Search Tool-nucleotide) on the NCBI website (www.ncbi.nlm.nih.gov) to confirm the correctness of the fish species. The BLASTn results presented in Table 1 show that of the nine samples observed, all are *Nomorhamphus liemi* species.

Table 1. Nucleotide base sequence validation results on NCBI website

Code	Identification	Accession
1a	*Nomorhamphus liemi*	LC153118.1
1b	*Nomorhamphus liemi*	LC153118.1
1c	*Nomorhamphus liemi*	LC153118.1
2a	*Nomorhamphus liemi*	LC153118.1
2b	*Nomorhamphus liemi*	LC153118.1
2c	*Nomorhamphus liemi*	LC153118.1
3a	*Nomorhamphus liemi*	LC153118.1
3b	*Nomorhamphus liemi*	LC153118.1
3c	*Nomorhamphus liemi*	LC153118.1

Alignment of the COI gene nucleotide sequence of *Nomorhamphus liemi*

Based on the results of the COI gene alignment in *Nomorhamphus liemi*, a nucleotide length of 645 bp was obtained. Nucleotide sequence alignment resulted in a nucleotide base composition consisting of 26.2% thymine base (T), 25.1% adenine base (A), 20.5% guanine base (G), and 28.3% cytosine base (C).

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Genetic distance

The genetic distance of COI gene fragments between *Nomorhamphus liemi* obtained from the three locations ranged from 0 to 0.03. Calculation of the interspecies genetic distance of *Nomorhamphus* spp. from showed a close relationship. The genetic distance between the samples and the outgroup ranged from 0.14 to 0.16. The greater the genetic distance of the test species, the lower the kinship.

Phylogeny analysis

A phylogeny tree of *N. liemi* was constructed based on the genetic distances of the nucleotide bases of the COI gene. The phylogeny tree in Figure 3 shows the relationship between *N. liemi* fish species and outgroup species. The ingroup species of *N. liemi* are closely related.

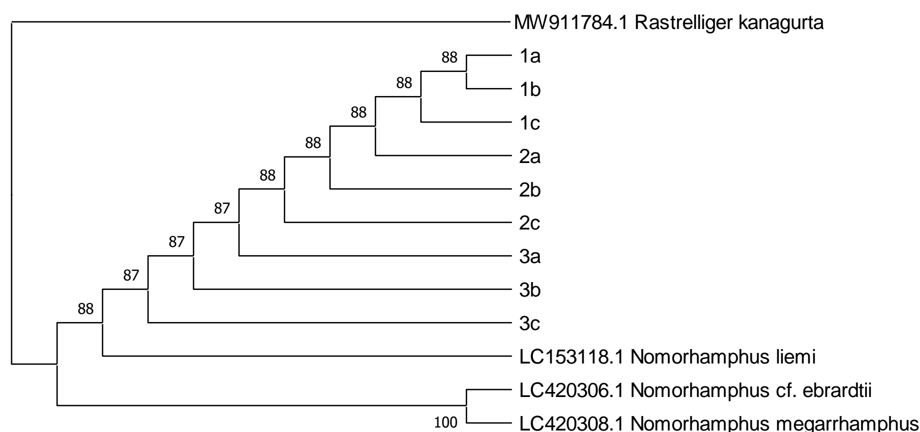

Figure 3. Construction of phylogeny tree based on COI gene

DISCUSSION

Molecular identification of angculung fish obtained from Sigi Regency, Central Sulawesi showed that there is one fish species, *N. liemi*. Total DNA from the isolation and extraction process of nine fish samples showed good quality. The DNA band seen on the electrophoresis machine showed no smear. This can be caused by the high concentration of DNA contained or not contaminated with RNA and protein (Barman et al. 2018) [6]. The presence of excessive debris and protein will inhibit the amplification process (Dahruddin et al. 2021) [15].

Amplification of *Nomorhamphus* spp. samples using PCR technique produced bright and clear DNA bands. Good total DNA quality will affect the results of amplification. In this study, the optimum temperature used in the primer attachment process (annealing) is 54ºC. This temperature is a suitable temperature for the amplification process because it produces good DNA bands. Good PCR products are very helpful in the sequencing process. The optimal annealing temperature is very important in the PCR method because the attachment of forward and reverse primers at both ends of the DNA occurs at the annealing stage. Too high a temperature can cause weak primer attachment and little DNA product is produced. This can result in visualization of thin DNA bands. Temperatures that are too low cause non-specific primer attachment and stick to any place. This causes the target region is not amplified but produces many non-specific products so that the visualization of thick DNA or smear (Dahruddin et al. 2021) [15].

The results of validation of mackerel on BLASTn showed that nine samples of mackerel from Sigi, Central Sulawesi showed the species *N. liemi* with sequence similarity of 98.04-99.65%. This indicates that the sample sequences are significantly similar to the GenBank database sequences. According to (Hutama et al., 2017) [7] the percentage similarity of sample sequences with GenBank database sequences is declared significant if the percentage value is between 97-100%.

Nucleotide sequence alignment of *Nomorhamphus* spp. resulted in a base composition dominated by adenine and thymine base bonds. Nucleotide sequence alignment resulted in a nucleotide base composition consisting of 26.2% thymine base (T), 25.1% adenine base (A), 20.5% guanine base (G), and 28.3% cytosine base (C). This is consistent with the results of studies in other species that show that the COI gene is rich in adenine and thymine. The A-T base bond consists of 2 hydrogen bonds and is weaker than the G-C base

bond which consists of 3 hydrogen bonds (Ng and Tan 2021). Therefore, the A-T nucleotide base bond is easier to separate, causing *Rastrelliger* spp. species to have a relatively high probability of mutation. However, despite the concept that the mutation rate in mitochondrial genes, especially A-T bases, is higher, the mutation rate of each part of the gene shows different rates (Barman et al. 2018) [6].

The nucleotide sequences of *Nomorhamphus* spp. underwent substitution mutations, namely transitions. A total of seven mutation sites were found in the nucleotide sequences of *N. liemi* species. This will affect the genetic distance of fish in that location. Genetic distance is determined by the bases that have changed. The more differences mean that the more frequent the mutation process occurs, which is indicated by the greater the genetic distance (Roesma et al. 2018) [15].

Based on the results of the pairwise distance method, the genetic distance of nine fish in the waters of the Sigi River, Central Sulawesi shows close kinship. The smaller the genetic distance value, the closer the kinship between species and vice versa. It can be concluded that the nine fish in the location are identical. The percentage of genetic distance can be used as information to determine the status of species, either the same species or different species (Hutama et al., 2017) [7].

The phylogenic tree showed that the nine fish from the three sites belonged to the same clade. Based on the clade formed, it can be concluded that there is mixing of mackerel populations in the three locations. The phylogeny tree also showed a clear separation between the *Nomorhamphus* genus present in the Sigi river, Central Sulawesi and the outgroup species obtained from GenBank. Outgroup species are used as correction factors in determining characters among ingroups (Dahruddin et al. 2021) [15]. The outgroup species used are species that are still in the same class and family.

According to (Barman et al. 2018) [6] information on species biology is needed to determine sustainable management strategies. An important first step is species identification. The information obtained from this molecular identification is the certainty of mackerel identity that can be used to support the study of other biological aspects such as stock assessment.

Mackerel in Sulawesi river waters has been widely caught in various sizes considering that the fish is the main commodity in community fisheries (Roesma et al. 2018) [15]. Increased catches can threaten the sustainability of mackerel in the waters. This can result in the mackerel population in the waters experiencing overfishing, and if not managed properly it will lead to extinction. The results of this study show the mixing of mackerel populations in three locations so that the species can be managed in one management unit. Management that can be carried out is the translocation of mackerel from healthy populations to overfished populations. Genetic information of angculung (*N. liemi*) needs to be studied further using other gene markers to obtain a complete genome. Research on the reproductive biology and population dynamics of angculung fish in the waters of the Sigi River, Central Sulawesi also needs to be conducted so that it can be used as a basis for determining appropriate mackerel management strategies in the region.

CONCLUSION

Our genetic identification showed that the phylogenetic investigation additionally settled that the examples that were dissectedare without a doubtendemic types of *N. liemi*. Finally, we expect there are future studies that pay attention to well-resolvedphylogenies andthereby remove additional noise from the analysis. The research data are expected to contribute to the preservation and utilization of one of Indonesia'simportant biodiversity resources.

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